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# INTERACTION AND PROTECTION OF ANTIMICROBIAL COMPOUNDS WITH CARBOHYDRATE-BASED COLLOIDAL SYSTEMS FOR IMPROVED FOOD SAFETY

PREETAM SARKAR

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CARBOHYDRATE-BASED COLLOIDAL SYSTEMS FOR IMPROVED FOOD SAFETY

For the degree of Doctor of Philosophy



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INTERACTION AND PROTECTION OF ANTIMICROBIAL COMPOUNDS WITH  
CARBOHYDRATE-BASED COLLOIDAL SYSTEMS FOR IMPROVED FOOD  
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of

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West Lafayette, Indiana

To: Major professor, Dr. Yuan Yao and my family.

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## ABSTRACT

Sarkar, Preetam. Ph.D., Purdue University, December, 2014. Interaction and protection of antimicrobial compounds with carbohydrate-based colloidal systems for improved food safety. Major Professor: Yuan Yao.

The food processing industry has focused on using conventional methods for food preservation. However, the recurring incidence of food borne illnesses and outbreaks due to pathogenic or spoilage organisms have called for creation of novel intervention strategies. In recent years, antimicrobial compounds from natural sources are gaining significant importance in improving food safety and security. However, antimicrobials suffer from extensive degradation when applied to complex food systems due to several specific or non-specific interactions. There is an urgent need for the protection of such antimicrobials to prolong their activity in foods. One strategy of achieving the goal is by the combination with a delivery vehicle. In this work, we explored the interaction between novel carbohydrate biomaterial and model antimicrobial compounds. In addition, we studied the protection of such antimicrobials using carbohydrate based colloidal systems. In the first part, we examine the interactions between nisin and carbohydrate based materials in non-emulsion (aqueous) and emulsion systems. Phytoglycogen octenyl succinate (PG-OS), a dendrimer-like amphiphilic material was used as the model carbohydrate nanoparticle and nisin was used as the model antimicrobial peptide. Equilibrium dialysis experiments showed that PG-OS with different degree of substitution (DS) interacts with nisin in a Langmuir monolayer adsorption pattern in both non-emulsion and emulsion systems. The monolayer adsorption capacity ( $Q_m$ ) increased in non-emulsion system and was significantly higher in the emulsion system.

At the equivalent concentration of PG-OS (5.0 mg/mL), and nisin (200 µg/mL), adsorbed nisin concentration in aqueous and emulsion systems were 22 and 157 µg/mL, respectively. The study demonstrated that DS of PG-OS can be used to control nisin adsorption in the non-emulsion system. In addition, the distribution of PG-OS at the droplet interface affects nisin adsorption in the emulsion system.

In the second part of the thesis, we studied the protection of nisin as model antimicrobial using starch octenyl succinate or starch-OS based oil-in-water emulsion system against *Listeria monocytogenes*. Cantaloupe juice was used as the model food system because of its significant protease concentration, which depletes nisin activity. Nisin was combined with starch-OS stabilized emulsion at 500 µg/mL initial concentration and compared with nisin solution in cantaloupe juice model. The study demonstrated that protease in cantaloupe juice was the key factor behind nisin depletion. Antimicrobial efficacy tests revealed that starch-OS stabilized emulsion can prolong nisin activity till 6 days of interaction with cantaloupe juice. In addition, equilibrium dialysis experiments demonstrated that nisin adsorption to the colloidal system was responsible for prolonged antibacterial activity. In the last part, the protection of nisin and thymol combined with starch-OS emulsion against a model Gram-positive bacteria (*Listeria monocytogenes*) and a model Gram-negative bacteria (*Salmonella* Typhimurium) in cantaloupe juice is reported. Thymol, being a volatile essential oil component suffers from extensive loss from foods due to evaporation, therefore it needs to be protected using a delivery vehicle. In general, combinations of nisin and thymol formulations demonstrated minor collaborative effects with regards to bacterial inhibition. Delivery system combined antimicrobials demonstrated effect till 7 days of incubation with cantaloupe juice. Out of all formulations, nisin and thymol combination with emulsion demonstrated the best antibacterial efficacy for a prolonged period of time. After 7 days of incubation, it showed 2.53 and 2.80 log<sub>10</sub> CFU/mL difference against *Listeria monocytogenes* and *Salmonella* Typhimurium, respectively. Overall, this research was an attempt to understand the interaction and extend the effect of antimicrobial molecules combined with carbohydrate materials as colloidal systems.

## CHAPTER 1. REVIEW OF LITERATURE

### 1.1 Food borne illnesses in USA

Disease outbreaks due to food borne pathogens are a major cause of concern. The Center for Disease Control (CDC) lists various causative agents of such illnesses including bacteria, fungus, parasites, and viruses. Pathogens, especially bacteria are responsible for causing a wide variety of infections and intoxications through contaminated foods that result in moderate to severe illness and deaths. In 2012, CDC estimated that 1 out of every 6 Americans or nearly 48 million gets sick due to food borne diseases. 128,000 are hospitalized and 3000 eventually die of such illnesses (Scallan, Hoekstra, Angulo, Tauxe, Widdowson, Roy, et al., 2011). These cases had been caused due to two major groups of food borne illnesses: (1) food borne pathogens and (2) unspecified agents.

The most important food borne pathogens causing illnesses annually include Norovirus (5,461,731), *Salmonella*-nontyphoidal (1,027,561), *Clostridium perfringes* (965,958), *Campylobacter* spp (845,024), and *Staphylococcus aureus* (241,148). The major pathogens causing food borne illness resulting in hospitalization include: *Salmonella* (nontyphoidal), Norovirus, *Campylobacter* spp, *Toxoplasma gondii* and *E. coli* (STEC) O157. The most important group of organisms causing deaths were *Salmonella*, *Toxoplasma gondii*, *Listeria monocytogenes*, Norovirus and *Campylobacter* spp. Therefore it is justified that protection of food systems against such pathogens deserves special attention.

## 1.2 Fresh produce industry in USA

Minimally processed fresh and fresh-cut produce is an important food processing sector due to a worldwide demand for healthier lifestyle. Fresh fruits and vegetables have shown promising results in the reduction for certain cancers and chronic illnesses including coronary heart disease (Goodburn & Wallace, 2013). Although fresh fruits and vegetables are excellent source of nutrients for the human body, they are significantly affected by pathogenic or spoilage microorganisms (Goodburn & Wallace, 2013; Lynch, Tauxe, & Hedberg, 2009). From 1990 to 2005, there were at least 713 outbreaks directly associated with fresh or fresh-cut produce in the United States (Goodburn & Wallace, 2013). The major groups of pathogens responsible for fresh produce contamination include *Salmonella* spp., *Shigella* spp., *E. coli*, *Campylobacter*, *Yersinia enterocolitica*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium* spp., *Bacillus cereus*, *Vibrio* spp., viruses and parasites (Goodburn & Wallace, 2013). Comprehensive measures need to be taken to preserve and protect produce crops against pathogens and spoilage organisms.

## 1.3 Cantaloupes and their safety issues

Melon (*Cucumis melo* L.) is one of the highly cultivated crops of the world, including the United States. Different types of melons have been cultivated including winter melon, honeydew melon, watermelon, muskmelon and cantaloupe melons. Among them, cantaloupes are the most popular choices in the United States (Laur & Tian, 2011). Cantaloupe belongs to the family of *Cucurbitaceae* and is one of the most popular fruits among American consumers. Cantaloupes are rich in  $\beta$ -carotene, vitamin A, B6, C and potassium (Alvarado-Casillas, Ibarra-Sanchez, Rodriguez-Garcia, Martinez-Gonzales, & Castillo, 2007; Gil, Aguayo, & Kader, 2006), which are known to possess anti-oxidant and anti-inflammatory properties (Solval, Sundararajan, Alfaro, & Sathivel, 2012; Vouldoukis, Lacan, Kamate, Coste, Calenda, Mazier, et al., 2004). On a daily value (DV) basis, one hundred gram of cantaloupe can provide 61.7% of vitamin C (36.7 mg) and

67.64% of vitamin A (3382 IU) (Solval, Sundararajan, Alfaro, & Sathivel, 2012). These biochemical properties indicate the popularity of cantaloupe melons among American consumers.

In the last decade, cantaloupes have been associated with a host of food borne pathogens including *Salmonella enterica* serovar Poona, *Salmonella* Chester, *Salmonella* Oranienburg, *Salmonella* Saphra, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Campylobacter* and Norovirus (CDC 2011; FDA 2010). The major sources of cantaloupe contamination with pathogens have been linked to microbes from soil (Islam, Morgan, Doyle, Phatak, Millner, & Jiang, 2004; Watkins & Sleath, 1981), irrigation water (Steele & Odumeru, 2004), washing water (Parnell, Harris, & Suslow, 2005), and animals (Parnell, Harris, & Suslow, 2005). They can also get contaminated during growth, harvesting, transportation, and distribution (Hedberg, MacDonald, & Osterholm, 1994; Parnell, Harris, & Suslow, 2005; Tauxe, 1997). The greatest outbreak in cantaloupe industry has been associated with *Listeria monocytogenes* in 26 states across US resulting in 130 illnesses and 30 deaths (Upadhyay, Upadhyaya, Mooyottu, Kollanoor-Johny, & Venkitanarayanan, 2014). In addition, *Salmonella* outbreak originated in Southwest Indiana leading to 261 illnesses with 94 hospitalizations and 3 deaths (CDC, 2012).

Whole cantaloupe possesses great risks for microbial contamination. Whole cantaloupe melons include a mesh like network on their surface known as “net” (Ukuku, 2006), creating an ideal surface architecture for bacterial attachment (Ukuku, 2006). In addition, the formation of bacterial biofilms on whole cantaloupe surface also reduces the efficacy of antimicrobial components by providing protection to the cells (Annous, Solomon, Cooke, & Burke, 2005). Similar to whole cantaloupes, fresh-cut cantaloupes also possess greater risk of pathogen contamination and public health concerns (Fang, Liu, & Huang, 2013; Luna-Guzmán & Barrett, 2000; Ukuku, 2006). Fresh-cut cantaloupes, like many other fruits contain significant amounts of juice which is rich in nutrients and enzymes (Fang, Liu, & Huang, 2013). In addition, the pH of cantaloupe juice ranges between 5.2 to 6.7, water activity between 0.95 and 0.98, and brix between 8.50 and 11.40 making it ideal candidate for bacterial growth (Galeb, Wrolstad, & McDaniel, 2002; Sharma, Adler, Harrison, & Beuchat, 2005; Wang, Ma, Zhao, Liao,



Chen, Wu, et al., 2006). Therefore, to preserve cantaloupes (whole or fresh-cut), novel preservation strategies needs to be developed and implemented.

#### 1.4 Current processing methods used in food preservation

##### 1.4.1 Thermal processing of foods

Thermal processing of foods for inhibiting spoilage and pathogenic micro-organisms have been extensively used for over a century. Strategies such as high temperature short time (HTST) processes and aseptic processes have been used successfully for preserving a wide range of food products. Thermal processing can affect physical and biochemical properties of a food material such as appearance (color, flavor, texture, and sound), nutritional properties (digestibility, availability, palatability, and delivery), functional properties (phase transition, density, volume, water content, solubility, and assembly), safety aspects (microorganisms, toxins, allergens) and quality parameters (enzyme properties, sensory, shelf life). Thermal processing have been applied to a number of fresh produce such as tomatoes (Dewanto, Wu, Adom, & Liu, 2002), whole cantaloupes (Annous, Burke, Sites, & Phillips, 2013), spinach (Gil, Ferreres, & Tomás-Barberán, 1999), bell pepper fruits (Castro, Saraiva, Lopes-da-Silva, Delgadillo, Loey, Smout, et al., 2008), and a host of other fruit and vegetables.

##### 1.4.2 High pressure processing of foods

High pressure processing is a method where food materials are subjected to pressures exceeding 100 MPa for several minutes at room temperature to inhibit microbial cells (San Martin, Barbosa-Canovas, & Swanson, 2002). High pressure treatments causes rapid damage to cell membranes that affects cell permeability, ion exchange capability, and inactivation of enzymes (Cheftel, 1995; Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007). High pressure treatment is generally used in foods where the use of high temperature can affect the material adversely. High hydrostatic pressure has found major applications in different food systems including

fruits and vegetables and their products including orange juice, mango pulp (Kaushik, Kaur, Rao, & Mishra, 2014), lemon juice, guava juice, apple juice, fresh cut pineapple, strawberry juice, asparagus, spinach, cauliflower and onions, white cabbage, and tomato puree (Ahmed, Ramaswamy, & Hiremath, 2005; Oey, Lille, Van Loey, & Hendrickx, 2008; Rastogi, Raghavarao, Balasubramaniam, Niranjana, & Knorr, 2007).

#### 1.4.3 Pulsed electric field processing

Pulsed electric field (PEF) is a novel method of food preservation where high voltage transient pulses (20 - 80 kV/cm) can pasteurize food products non-thermally (Barsotti & Cheftel, 1999). PEF processing exhibits the advantage of greater preservation of color, flavor, texture and nutrition of foods as opposed to thermal treatments (Barsotti & Cheftel, 1999). PEF processing has been greatly successful with liquid or emulsion based foods such as apple and orange juice (Buckow, Ng, & Toepfl, 2013; Cortés, Esteve, & Frígola, 2008; Evrendilek, Zhang, & Richter, 2004), milk (Evrendilek, Zhang, & Richter, 2004; Sepulveda, Góngora-Nieto, Guerrero, & Barbosa-Cánovas, 2005; Shamsi, Versteeg, Sherkat, & Wan, 2008), egg and egg products (Amiali, Ngadi, Smith, & Raghavan, 2007; Bazhal, Ngadi, Raghavan, & Smith, 2006), green pea soup (Vega-Mercado, Martín-Belloso, Qin, Chang, Marcela Góngora-Nieto, Barbosa-Cánovas, et al., 1997) and to a lesser extent in powders or solid foods.

#### 1.4.4 Plasma processing of foods

Non-thermal plasma is defined as the fourth state of matter and is concurrent with higher energy state of ionized gases. In food processing conditions, it is also called cold or non-thermal plasma as it is operated in ambient temperatures. Different types of technologies have been developed based on non-thermal plasma such as remote treatment/enclosed chamber strategies, direct applications and in-package applications. Cold plasma technology has been used for the inactivation of pathogenic bacteria in fresh produce such as apples, tomatoes (Kim, Yun, Jung, Jung, Jung, Choe, et al., 2011), and

strawberries (Pankaj, Bueno-Ferrer, Misra, Milosavljević, O'Donnell, Bourke, et al., 2014).

#### 1.4.5 Food irradiation

Irradiation is the process of treating food materials with a specific dosage of ionizing radiation for preservation purposes. Three major types of irradiation, electron beam, x-ray and gamma rays have found applications within food processing industries (Farkas, 1998). In electron beam irradiation systems, electrons are accelerated with strong magnetic fields. X-ray irradiation is generated when high intensity electrons hit against heavy metals such as tungsten. Gamma irradiation is created by the radioactive reactions of  $^{60}\text{Cobalt}$  or  $^{137}\text{Cesium}$ . The US FDA has approved the use of irradiation on certain varieties of fresh produce including lettuce, spinach, and fresh fruits (Pillai, Bogran, & Blackburn, 2014).

It has been used for the inhibition of food borne pathogenic bacteria including *Campylobacter*, *Salmonella* and *Escherichia coli* O157:H7 and delay the growth of spoilage organisms (Farkas, 1998). Gamma irradiation has been utilized for the inactivation of *Salmonella* Typhimurium in peanut butter (Ban & Kang, 2014). X-ray irradiation had been applied successfully to chemically injured spinach leaves contaminated by *Escherichia coli* O157:H7 (Moosekian, Jeong, & Ryser, 2014). Gamma irradiation in combination with antimicrobial coatings have been used in broccoli florets for inhibition of pathogens including *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella* Typhimurium (Moosekian, Jeong, & Ryser, 2014).

#### 1.4.6 Antimicrobial compounds

Antimicrobial compounds are defined as agents that prolong the shelf life of foods. They can be extracted from plants, animals and microbes (Tiwari, Valdramidis, O'Donnell, Muthukumarappan, Bourke, & Cullen, 2009). Plants are known to generate a host of chemical compounds and their byproducts that show strong antimicrobial efficacy against bacteria, yeasts and molds. Some of the common plant based antimicrobials include essential oil components such as thymol, eugenol, carvacrol and trans-

cinnamaldehyde. Antimicrobials are also extracted from animal sources such as defensins, pleurocidin, lactoferrin, chitosan, and certain free fatty acids (Tiwari, Valdramidis, O' Donnell, Muthukumarappan, Bourke, & Cullen, 2009). Antimicrobials from microbes include peptides such as bacteriocins (nisin and pediocin) and reuterin (Tiwari, Valdramidis, O' Donnell, Muthukumarappan, Bourke, & Cullen, 2009).

## 1.5 Overview of natural antimicrobial compounds

### 1.5.1 Antimicrobial peptides and nisin

In the early 1950's, antimicrobial peptides (AMP) were first extracted from natural sources. Antimicrobial peptides can be found in a variety of plants, animals and micro-organisms. Bacteriocins, secreted by both Gram positive and negative organisms are proteins which are synthesized ribosomally (Cleveland, Montville, Nes, & Chikindas, 2001). Bacteriocins are classified into three major groups. Class I bacteriocins, also known as lantibiotics, are smaller peptides (< 5 kDa) which contain certain unusual amino acid residues such as lanthionines and  $\beta$ -methyl lanthionine rings (Cleveland, Montville, Nes, & Chikindas, 2001; Oscáriz & Pisabarro, 2001). Class I bacteriocins contains nisin, which has been approved by the US FDA for use as food additive (Cotter, Hill, & Ross, 2005). Class II bacteriocins are non-lanthionine containing peptides which exhibit heat resistance (< 10 kDa). Class II bacteriocins are divided into three sub-families, out of which 2a is the most important class (includes the peptide pediocin) (Oscáriz & Pisabarro, 2001). In addition, class III bacteriocins are a group of large heat-labile bacteriocins (> 30 kDa). Class III bacteriocins contains peptides that can behave as antibiotics and mostly produced by *Lactobacillus* group (Oscáriz & Pisabarro, 2001). Sometimes, class IV bacteriocin is also defined which contain molecules with both carbohydrate and lipid fractions (glycoproteins or lipoproteins), which contain complex cyclic molecules (Oscáriz & Pisabarro, 2001).

Nisin is produced by *Lactococcus lactis* via fermentation process. Nisin is a polycyclic, cationic antibacterial peptide comprising of 34 amino acid residues

(molecular weight: 3.4 kDa) (Breukink, Ganz, de Kruijff, & Seelig, 2000; de Arauz, Jozala, Mazzola, & Penna, 2009). Nisin is also known as a lantibiotic because it contains certain unusual amino acid residues such as lanthionine and  $\beta$ -methyl lanthionine (Breukink, Ganz, de Kruijff, & Seelig, 2000; de Arauz, Jozala, Mazzola, & Penna, 2009). These unusual amino acids are responsible for nisin's heat stability, activity at lower pH conditions and a unique mechanism of antibacterial action (Breukink, Ganz, de Kruijff, & Seelig, 2000; de Arauz, Jozala, Mazzola, & Penna, 2009). It has been found to be highly effective against gram positive bacteria such as *Listeria monocytogenes* (Breukink & Kruijff, 1999; Arauz, Jozala, Mazzola, & Penna, 2009). Nisin kills bacteria by forming pores on cell membranes. Nisin is the only FDA approved GRAS additive which is extensively used in the food processing industry (Breukink, Ganz, de Kruijff, & Seelig, 2000; de Arauz, Jozala, Mazzola, & Penna, 2009).

Studies have confirmed that nisin can enhance the permeability of bacterial cell membranes by two different mechanisms: (a) low affinity system and (b) highly efficient system (Breukink & de Kruijff, 2006). In the low affinity mechanism, nisin interacts with the anionic lipids and phospholipid groups present on the bacterial cell membrane. Further, nisin accumulation within the phospholipid groups drives the formation of transient pore-like structures. In the second mechanism, nisin interacts specifically with lipid II molecules of the Gram-positive bacterial cell membrane. After binding to cell surface, nisin accumulate and assemble to form pore (Breukink & de Kruijff, 2006; Breukink, Ganz, de Kruijff, & Seelig, 2000; de Arauz, Jozala, Mazzola, & Penna, 2009). Even though highly effective against Gram-positive organisms, nisin faces severe depletion when applied in foods. Different food components (for example, enzymes, glutathione, sodium metabisulfite and titanium dioxide) can significantly decrease nisin activity by specific or non-specific interactions (Bhatti, Veeramachaneni, & Shelef, 2004; Jung, Bodyfelt, & Daeschel, 1992; Mahadeo & Tatini, 1994; Rose, Sporns, Stiles, & McMullen, 1999). In addition, nisin can also interact with hydrophobic food components such as lipids and proteins which reduces its effectiveness.

### 1.5.2 Essential oils and thymol

Essential oils, also known as volatile or ethereal oils, are obtained from different sections of the plant including flowers, buds, seeds, barks, herbs, fruits and roots. Today, there are about 3000 essential oils that are known globally, but out of them, 300 have been well characterized and are important industrially (Burt, 2004). Essential oil components demonstrate different biological activities including anti-inflammatory, expectorant property, psychoactivity, and antimicrobial activity (Saad, Muller, & Lobstein, 2013). The antibacterial mechanism and cellular targets of essential oil components vary greatly due to the differences in their molecular structures and physico-chemical properties (Lambert, Skandamis, Coote, & Nychas, 2001; Skandamis & Nychas, 2001). Studies have confirmed that essential oil components with a higher proportion of phenolic compounds generally exhibit stronger antibacterial effects (Cosentino, Tuberoso, Pisano, Satta, Mascia, Arzedi, et al., 1999; Dorman & Deans, 2000; Juliano, Mattana, & Usai, 2000; Lambert, Skandamis, Coote, & Nychas, 2001; Thoroski, Blank, & Biliaderis, 1989). Some examples of phenol rich compounds include thymol, carvacrol and eugenol. The most common mechanism of their antibacterial effects is due to their hydrophobic character. Essential oil components can penetrate within the cell and mitochondrial membrane structures, leading to leakage of the membrane, disruption of proton motive force, and excessive loss of critical ions, molecules and cell death (Carson, Mee, & Riley, 2002; Cox, Mann, Markham, Bell, Gustafson, Warmington, et al., 2000; Gustafson, Liew, Chew, Markham, Bell, Wyllie, et al., 1998; Helander, Alakomi, Latva-Kala, Mattila-Sandholm, Pol, Smid, et al., 1998; Knobloch, Pauli, Iberl, Weigand, & Weis, 1989; Lambert, Skandamis, Coote, & Nychas, 2001; Oosterhaven, Poolman, & Smid, 1995; Sikkema, de Bont, & Poolman, 1995; Skandamis & Nychas, 2001; Ultee, Bennik, & Moezelaar, 2002).

Thymol (2-isopropyl-5-methylphenol) is a major monoterpene phenol obtained from thyme (*Thymus vulgaris*) essential oil. According to (FAO/WHO, 2008), thymol is considered as generally recognized as safe (GRAS). The antibacterial efficacy of thymol is primarily due to the presence of the hydroxyl group in their phenolic structure. In case of Gram-negative bacterial cell membrane, thymol targets the outer membrane containing

lipopolysaccharides (LPS) and thus enhances permeation of ATP. In the case of Gram-positive bacterial cell membrane, thymol aligns itself within the fatty acids of the phospholipid membrane. This results in disintegration of the membrane structures and leads to increased cellular permeability (Helander, et al., 1998; Lambert, Skandamis, Coote, & Nychas, 2001; Ultee, Bennik, & Moezelaar, 2002).

Thymol faces extensive depletion in food systems due to several factors. First, thymol, being a hydrophobic molecule, can interact with lipophilic food components such as proteins and lipids, which can reduce its effective available concentration. Second, thymol being volatile in nature is lost due to evaporation. Third, the maximum solubility of thymol in water phase is about 1000 µg/mL at 25 °C, therefore sufficient concentrations may not be achieved to be effective against food borne micro-organisms. Fourth, thymol exhibits a strong medicinal aroma at elevated concentrations that can limit its application in foods due to sensory issues (Guarda, Rubilar, Miltz, & Galotto, 2011; Liolios, Gortzi, Lalas, Tsaknis, & Chinou, 2009; B. Shah, P. M. Davidson, & Q. Zhong, 2012; Weiss J, 2009). Therefore, there is a genuine need to improve thymol functionality when applied in real food systems.

## 1.6 Delivery systems for antimicrobial molecules in foods

### 1.6.1 Delivery systems

Delivery systems are carriers that allows for the protection of the active ingredient against different depletion factors such as oxidation, precipitation, crystallization, denaturation, light, temperature, pH, moisture, ionic strength variations, adsorption, diffusion, and biochemical interactions (Augustin & Hemar, 2009; McClements, Decker, Park, & Weiss, 2009). It should also allow for the prolonged or controlled release of the active molecule when desired at the targeted site of delivery due to certain trigger mechanisms such as changes in pH, ionic strength or enzyme activity (Augustin & Hemar, 2009; McClements, Decker, Park, & Weiss, 2009). Considering the structural and physico-chemical aspects of delivery systems, they can be broadly classified into three

major categories: (a) lipid based delivery vehicles, and (b) solid-particle based delivery vehicles, and (c) film based delivery vehicles.

### 1.6.2 Lipid based delivery systems

Some of the major lipid-based carrier systems include emulsions [conventional emulsions, multiple emulsions and multilayered emulsions], liposomes, and solid lipid nanoparticles. Conventional emulsions consist of two immiscible phases, oil and water with one system being dispersed in the other in the form of small droplets. Based on their spatial distribution, they can be classified into oil-in-water (O/W) or water-in-oil (W/O) emulsions (McClements, Decker, Park, & Weiss, 2009). Multiple emulsion systems such as water-in-oil-in-water (W/O/W) can be prepared by water droplets in oil phase and then dispersing them in an aqueous continuous phase. Similarly, oil-in-water-in-oil (O/W/O) systems can also be prepared. Multi-layered emulsions can be created by the interaction between different layers of emulsifiers of opposite electrostatic charge. This method of layer-by-layer deposition of emulsifying agents on the droplet surface provides high stability to the active molecules (McClements, Decker, Park, & Weiss, 2009).

Emulsion systems have been used for the formulation, protection and delivery of different antimicrobial compounds for food preservation. For example, oil-in-water emulsion systems have been used for the encapsulation of essential oil components from different plant sources such as lemongrass, clove, thyme and palmarosa (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso). Emulsion systems have also been used for the protection of tributyl phosphate and n-pentanol against *S. aureus* NCTC 1803, *L. monocytogenes* and *E. coli* O157:H7 (Ferreira, Alves, Neves, Silva, Gibbs, & Teixeira, 2010). Oil-in-water emulsions have been used for the protection and delivery of carvacrol (Donsì, Cuomo, Marchese, & Ferrari, 2014), thyme oil (Wu, Lin, & Zhong, 2014), mixture of terpenes and D-limonene (Donsì, Annunziata, Sessa, & Ferrari, 2011), and eugenol (Suriyarak & Weiss, 2014). Multiple emulsion (oil-in-water-in-oil) system have been created for the protection of essential oil component from summer savory (*Satureja hortensis*) against *Staphylococcus aureus* (Hosseini, Hosseini, Mohammadifar, Mortazavian, Mohammadi, Khosravi-Darani, et al., 2013).



Liposomes are lipid vesicles formed from polar lipids that produce bilayer structures (Taylor, Davidson, Bruce, & Weiss, 2005). They are spherical in shape and range from nanometer to micrometer in dimensions. Liposomes contain a single or multi-layer of amphiphilic membranes. Liposomes with single layer of membranes are known as single (<30 nm) or large unilamellar vesicles (30-100 nm) (SUV or LUV). Liposomes with multilayer membranes are classified as multilamellar vesicles (MLV) or multivesicular vesicles (MVV) (Taylor, Davidson, Bruce, & Weiss, 2005). Liposomes have been used extensively for the protection and delivery of antimicrobials, fat soluble vitamins, enzymes and minerals (Taylor, Davidson, Bruce, & Weiss, 2005). Liposomes have been used for the encapsulation and delivery of different antimicrobials such as peptides (nisin, pediocin), and essential oil components (carvacrol, thymol) (Benech, Kheadr, Lacroix, & Fliss, 2003; Degnan, Buyong, & Luchansky, 1993; Kopermsub, Mayen, & Warin, 2011; Laridi, Kheadr, Benech, Vuillemand, Lacroix, & Fliss, 2003; Liolios, Gortzi, Lalas, Tsaknis, & Chinou, 2009; Malheiros, Sant'Anna, Barbosa, Brandelli, & Franco, 2012).

Solid lipid nanoparticles (SLN's) are colloidal delivery systems that comprise a core of solid lipid which incorporates lipid based bioactive ingredients. SLN's are manufactured by a homogenization process to form an emulsion of liquid lipids and an aqueous solution of surfactants at a temperature greater than the melting temperature. The emulsion is further cooled below the crystallization temperature to obtain solid lipid nanoparticles. They have been considered as an alternative to conventional polymeric nanoparticles. It consists of a core lipid which is stabilized by a single or mixed layer of surfactants. SLN's combine the functional advantages of polymeric nanomaterials, liposomes, and emulsion systems. For example, the use of solid lipid structures instead of liquids help to achieve controlled delivery of functional components and enhance the stability of such compounds by reducing the rate of chemical reactions such as oxidation. SLN's have found applications in the protection and delivery of bioactives such as  $\beta$ -carotene (Qian, Decker, Xiao, & McClements, 2013),  $\alpha$ -tocopherol (de Carvalho, Noronha, Floriani, Lino, Rocha, Bellettini, et al., 2013), nisin (Prombutara, Kulwatthanasal, Supaka, Sramala, & Chareonpornwattana, 2012), resveratrol (Pandita,

Kumar, Poonia, & Lather, 2014), quercetin (Aditya, Macedo, Doktorovova, Souto, Kim, Chang, et al., 2014) and  $\omega$ -3 fatty acid (Salminen, Helgason, Kristinsson, Kristbergsson, & Weiss, 2013). Interestingly, SLN's have also been used for the preparation of edible films for protection of fruits such as guava (Zambrano-Zaragoza, Mercado-Silva, Ramirez-Zamorano, Cornejo-Villegas, Gutiérrez-Cortez, & Quintanar-Guerrero, 2013).

#### 1.6.2.1 Particle stabilized emulsions as delivery systems

Pickering emulsions are defined as oil-in-water, water-in-oil or multiple emulsions stabilized by solid particles and not by surfactants (Binks & Lumsdon, 2001; Chevalier & Bolzinger, 2013). Pickering emulsions retain the fundamental properties of conventional emulsions but show extended stability (Aveyard, Binks, & Clint, 2003; Chevalier & Bolzinger, 2013). Pickering emulsions provide greater advantages compared to conventional emulsions: (1) they are more resistant to emulsion destabilization mechanisms such as coalescence, flocculation and Ostwald ripening, and (2) they are prepared without surfactants which approves their usage in different fields (Aveyard, Binks, & Clint, 2003; Chevalier & Bolzinger, 2013). Solid particles have been used for the protection and release of bioactives including antimicrobial compounds such as pea protein isolate (Liang & Tang, 2014), phytoglycogen octenyl succinate (Bi, Yang, Bhunia, & Yao, 2011b), lactoferrin nanoparticles (Shimoni, Shani Levi, Levi Tal, & Lesmes, 2013) and microfibrillated cellulose (Winuprasith & Suphantharika, 2013). Therefore, there is a growing interest in the use of Pickering emulsions for the protection and delivery of functional molecules in food systems.

#### 1.6.3 Solid particle based delivery systems

Solid particle delivery systems include chitosan based micro or nanoparticles and phytoglycogen (PG) nanoparticles. Chitin and chitosan are extracted from the exoskeleton of crustaceans (crabs, shrimps, prawns, lobsters and fungi including *Aspergillus*, *Zygomycetes* and *Mucor*) (Pedro, Cabral-Albuquerque, Ferreira, & Sarmiento, 2009; Sinha, Singla, Wadhawan, Kaushik, Kumria, Bansal, et al., 2004). Chitin is a linear polysaccharide of  $\beta$ -(1-4)-2-acetamido-2-deoxy-D-glucopyranose monomeric units

(Sinha, et al., 2004). Chitosan, a derivative of chitin is a co-polymer of N-acetyl-D-glucosamine and D-glucosamine. Chemically, it is comprised of  $\beta$ -(1-4)-2-amino-2-deoxy-D-glucopyranose as the monomer. It is obtained from the alkaline deacetylation process of chitin. Chitosan is non-toxic, biodegradable, and biocompatible material that shows antimicrobial activity within biological systems. Therefore, it has found enormous applications within diverse fields such as foods, biotechnology, pharmaceuticals and gene therapy (A.-C. Lee & Hong, 2009). Chitosan possesses positive charge under acidic conditions and can therefore bind with negatively charged molecules (A.-C. Lee & Hong, 2009). Chitosan has been used extensively in the protection and prolonged delivery of different bioactive antimicrobial components such as EGCG (Dube, Ng, Nicolazzo, & Larson, 2010), curcumin (Akhtar, Rizvi, & Kar, 2012), organic acids (Rivero, Giannuzzi, García, & Pinotti, 2013). Chitosan has been used in different forms for the extension of shelf-life of fresh fruits and vegetables. For example, chitosan containing rosemary extracts has been coated on fresh-cut pears (C. Xiao, Zhu, Luo, Song, & Deng, 2010). Chitosan has also been applied with sodium chloride on fresh-cut pears (Z. Xiao, Luo, Luo, & Wang, 2011). Chitosan has been combined with methyl-cellulose to form edible coatings for pineapples and fresh-cut cantaloupes for the protection and delivery of vanillin (Sangsuwan, Rattanapanone, & Rachtanapun, 2008). Chitosan has been combined with essential oil components and their antibacterial efficacy tested against *Escherichia coli* and *Listeria monocytogenes* in fresh cut broccoli (Alvarez, Ponce, & Moreira, 2013).

Phytoglycogen is water soluble, glycogen like  $\alpha$ -D glucan obtained from the kernel of the maize mutant *su1*. Chemical modifications such as octenyl succinylation can be performed to phytoglycogen to impart useful functional properties. This modification results in the formation of phytoglycogen octenyl succinate (PG-OS). PG-OS stabilized oil/water emulsion system has been used for the protection of  $\omega$ -3-fatty acids in fish oil against lipid oxidation (Scheffler, Wang, Huang, San-Martin Gonzalez, & Yao, 2009). PG-OS nanoparticles and PG-OS stabilized oil/water emulsions have also been used for the prolonged protection and activity retention of antimicrobial peptide

such as nisin in brain heart infusion (BHI)-agar deep-well model system (Bi, Yang, Bhunia, & Yao, 2011b; Bi, Yang, Narsimhan, Bhunia, & Yao, 2011).

#### 1.6.4 Film based delivery systems

Film based delivery systems have been used extensively in food processing. Films have been prepared from biopolymers including carbohydrates, proteins, lipids and their combination. In addition, plasticizers are added to functionalize individual biopolymers. Films have been utilized for the protection and controlled delivery of different bioactive components including flavors, aroma, antimicrobials, probiotics, and antioxidants. Carbohydrate based films from starch, cellulose, pectin, guar gum, chitosan, alginate, galactomannans and cyclodextrins have found numerous applications in incorporation of active ingredients (Fathi, Martín, & McClements). Proteins have also found a lot of applications in film based delivery of bioactives. Proteins such as collagen, gelatin, fish myofibrillar proteins, keratin, egg white protein, casein, whey proteins, corn zein, wheat gluten, rice bran protein, soy protein, and peanut protein have been used for the encapsulation and delivery of functional molecules. Certain lipid based materials have also been used as film forming agents such as waxes, and emulsifiers or surface active agents (Morillon, Debeaufort, Blond, Capelle, & Voilley, 2002). Edible films and coatings from soy, water chestnut-chitosan complex, carboxy methyl cellulose, starch and pectin have been used for the delivery of different antimicrobials such as carvacrol, methyl cinnamate and thymol in ground beef patties (Emiroğlu, Yemiş, Coşkun, & Candoğan, 2010; Peretto, Du, Avena-Bustillos, Sarreal, Hua, Sambo, et al., 2014), potassium sorbate in fresh pistachios (Sayanjali, Ghanbarzadeh, & Ghiassifar, 2011), nisin and natamycin in cheese systems (Ollé Resa, Gerschenson, & Jagus, 2014), oregano essential oil in chicken breast fillets (Fernández-Pan, Carrión-Granda, & Maté, 2014), combinations of *Cornus officinalis* fruit extract, nisin, glycerol monolaurate, and pine cone essential oil (Mei, Yuan, Guo, Wu, Li, & Yu, 2013).

## 1.7 Delivery systems for peptides and phenolic compounds

### 1.7.1 Delivery systems for nisin

Nisin has been used as the major antimicrobial agent in combination with different types of delivery vehicles including nanoparticles, liposomes, emulsions, edible films and coatings and biopolymers as will be discussed in detail in this section.

Nisin was combined with solid lipid nanoparticles (SLN) using high pressure homogenization procedure. The study showed that the nisin loaded SLN's had a higher particle size when compared with unloaded nisin. The size for nisin loaded SLN ranged between 159 to 167 nm, the zeta-potential values -28.3 to -29.2 mV, nisin entrapment capability ranged between 69.2 to 73.6 %. When tested against *Listeria monocytogenes* DMST 2871 and *Lactobacillus plantarum* TISTR 850, the antibacterial effect lasted for 20 and 15 days, respectively, whereas for free nisin, antibacterial efficacy was positive for 2 to 3 days (Prombutara, Kulwatthanasal, Supaka, Sramala, & Chareonpornwattana, 2012).

Nanoliposomes prepared from different types of lipids, stearylamine, diacetyl phosphate and cholesterol were associated with nisin Z and their antibacterial efficacies were studied against *Bacillus subtilis* and *Pseudomonas aeruginosa*. Nisin entrapment efficacy ranged between 12 and 54% for the different lipid preparations. The study confirmed that the use of anionic lipids possessed the maximum power to bind nisin whereas with increase in cholesterol concentration, there was significant reduction in nisin entrapment. The stability of the preparations ranged between 12 months at 25 °C to 14 months at 4 °C (Colas, Shi, Rao, Omri, Mozafari, & Singh, 2007).

In another similar study, nisin was incorporated with liposomal nanoparticles, and their efficacies were tested against *Listeria monocytogenes* and *Staphylococcus aureus*. The particle size ranged between 114 and 125 nm. The loading capacity of nisin attained 70% and that resulted in a zeta-potential value of + 17.1 mV. After 48 and 72 h of incubation, both *Listeria monocytogenes* and *Staphylococcus aureus* were reduced by 6 logs (Zou, Lee, Seo, & Ahn, 2012).

Nisin was co-encapsulated with thymol in spray-dried zein nano-capsules along with glycerol and their effectiveness was studied against *Listeria monocytogenes* in a milk model. The study confirmed that nisin from zein capsules was bactericidal for *Listeria* cells at 30 °C and pH 6.0. The study also showed that the inhibition of *Listeria* was more effective when nisin was combined with zein capsules in the reduced fat milk system (D. Xiao, Davidson, & Zhong, 2011). In a similar study by the same group of authors, nisin was encapsulated in spray dried zein capsules using four different inlet drying temperatures ranging between 75 to 120 °C. It was concluded that above 95 °C, there was no loss of nisin activity. Also, pH 2.0 was more effective for nisin release that occurred in 30 min compared to pH 8.0 when it took 8 h for nisin release. 400 IU/mL of encapsulated nisin exhibited the strongest antibacterial efficacy against *Listeria monocytogenes* compared to free nisin in 2% fat milk (D. Xiao, Gommel, Davidson, & Zhong, 2011).

Nisin was complexed into palmitoylated alginate films and alginate beads and their efficacy against *Staphylococcus aureus* was studied. Then this nisin containing film based preparation was used to coat beef at three nisin concentrations: 0, 500 and 1000 IU/mL. When alginate film containing nisin was used at 4 °C, there was 0.91 and 1.86 log reduction in CFU/cm<sup>2</sup> after 7 days of storage, and 2.2 and 2.81 log reduction after 14 days of storage, when nisin concentrations were 500 and 1000 IU/mL, respectively. In the case of activated alginate beads, a 1.77 and 1.93 log reduction was achieved for 500 and 1000 IU/mL nisin concentration, respectively (Millette, Le Tien, Smoragiewicz, & Lacroix, 2007).

In another study, konjac glucomannan based films containing chitosan and nisin in different proportions were tested against *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus*. The highest tensile strength reported for the film combinations was 102.8 MPa and a strong water solubility and water vapor transmission ratio. Nisin at a concentration of 463 IU per disk showed strong antimicrobial effect against *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus*. The group concluded that konjac glucomannan based films with chitosan and nisin can effectively be used as a bioactive packaging material for future applications (B.

Li, Peng, Yie, & Xie, 2006). In another study, nisin was incorporated within an extruded film made of pectin and polylactic acid polymers (PLA). The study demonstrated that when pectin was combined with PLA containing nisin at 1000 IU/mL, there was about 2.1 to 4.5 log reduction in different medium such as Brain Heart Infusion, liquid egg white system and orange juice model (T. Jin, L. S. Liu, H. Zhang, & K. Hicks, 2009).

### 1.7.2 Delivery systems for thymol

Thymol and carvacrol were encapsulated within liposomes prepared from phosphatidylcholine, and tested against a host of Gram positive [*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus mutans* and *Staphylococcus viridans*] and Gram negative bacteria [*Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter cloacae* and *Klebsiella pneumoniae*] and three fungi [*Candida albicans*, *Candida tropicalis* and *Candida glabrata*] which are pathogenic to humans. Pure thymol and carvacrol components exhibited stronger antimicrobial efficacy compared to the oil, whereas liposomal encapsulation enhanced antimicrobial efficacy significantly as measured through agar diffusion assay (Liolios, Gortzi, Lalas, Tsaknis, & Chinou, 2009).

Nano-dispersed thymol (complexed in whey protein isolate and maltodextrin matrix) was tested against a host of food borne pathogens including *Escherichia coli* O157:H7 strains ATCC 43889 and 43894, *Salmonella* Typhimurium strain 2576, *Listeria monocytogenes* strains Scott A and 101, and *Staphylococcus aureus* strains 27708 and SA113 in a tryptic soy broth system. The minimum inhibitory concentration (MIC) of thymol against all the pathogens was found to be 500 µg/mL and most of the pathogens were inhibited at 300 µg/mL of free thymol. Tryptic soy broth at pH 5.5 showed complete inhibition of *Listeria* cells when treated with nano-dispersed thymol at 500 µg/mL, whereas there was about 1 to 3 log reduction in *Escherichia coli* O157:H7 cells (B. Shah, Davidson, & Zhong, 2013).

Zein nanoparticles prepared from sodium caseinate and chitosan hydrochloride were loaded with thymol, and their physico-chemical and antimicrobial properties were studied. Chitosan hydrochloride enhanced the hydrodynamic radius, decreased zeta-

potential value and increased its entrapment efficiency significantly. [Thymol + zein nanoparticles] and [sodium caseinate + zein nanoparticles] showed spherical shape under TEM. The free thymol and encapsulated thymol in sodium caseinate stabilized zein nanoparticles were tested at a concentration of 0.052 mg/ml, while for encapsulated thymol in chitosan hydrochloride-sodium caseinate zein nanoparticles, the concentration was 0.020 mg/ml. The study confirmed that the entrapped thymol was able to reduce bacterial counts for Gram positive strains more effectively (Zhang, Niu, Luo, Ge, Yang, Yu, et al., 2014).

Thymol was combined with  $\beta$ -cyclodextrin matrix as inclusion complex by two different methods, kneading and freeze drying. It was found that thymol was loaded between 71 and 83 g/100g of the cyclodextrin. The cyclodextrin combined thymol (freeze dried samples) demonstrated the best antimicrobial efficacy at log difference of 0.48, and 0.68 on day-2 and day-5, respectively (Tao, Hill, Peng, & Gomes, 2014). Thymol was combined with chitosan with different degree of deacetylation. The study reported that thymol chitosan micelles showed greater antimicrobial efficacy against two different bacteria, *Staphylococcus aureus* and *Bacillus subtilis* when compared to thymol alone samples (Hu, Du, Wang, & Feng, 2009). Thymol was added to zein-sodium caseinate based films and its antibacterial efficacy was tested against *Escherichia coli* and *Salmonella*. The antibacterial performance was tested using an agar diffusion assay technique. The ring sizes ranged between 15.89 to 18.81 mm and 16.02 to 18.12 mm *E. coli* and *Salmonella*, respectively (K.-K. Li, Yin, Yang, Tang, & Wei, 2012).

## 1.8 Commercially available delivery technologies

With the demand for healthier, convenient, nutritious, safe, and wholesome food choices, the use of delivery systems is increasing day by day (Perez & Gaonkar, 2014). Currently different types of food systems are available in the global market using delivery science rendering functionalities such as flavor, taste, texture and interactive packaging solutions. Beverage emulsions such as coffee, tea, juice and soups use delivery vehicles for the protection of thiols and esters.  $\omega$ -3-fatty acids, probiotics and prebiotics



are being incorporated within beverages, nutritional bars and cereal products using controlled delivery systems. A major class of nutritional products such as chewing gums use delivery systems for the protection of release of flavors, aromas, fruit volatiles. In addition, dairy systems such as cheese use ripening enzymes and health benefiting cultures (Perez & Gaonkar, 2014).

## 1.9 Starch and phytoglycogen based materials

### 1.9.1 Starch and starch octenyl succinate

Starch is the homopolymer of glucose. It has two components: (a) linear component known as amylose and (b) branched component known as amylopectin. Amylose has an average molecular weight of  $10^6$  whereas amylopectin has an average molecular weight of  $10^8$  (Sweedman, Tizzotti, Schafer, & Gilbert, 2013). Starch is the most important form of energy storage in plant cells. Native starches show limited functional properties during industrial applications. Modification of starch with groups including acetate, succinate or octenyl succinic anhydride is generally required to enhance their functional attributes. Octenyl succinic anhydride (OS) modification imparts hydrophobicity to starch. This results in the formation of a more amphiphilic molecule which can be used for different industrial applications such as emulsification, encapsulation, films/coatings, and gel production (Sweedman, Tizzotti, Schafer, & Gilbert, 2013).

### 1.9.2 Phytoglycogen and phytoglycogen octenyl succinate

Phytoglycogen is water soluble, glycogen like  $\alpha$ -D glucan obtained from the kernel of the maize mutant *su1*. This *su1* mutation results in the deficiency of SU1, a starch debranching enzyme (DBE). The major function of starch DBE is to cut off the abnormal branches that retard the formation of starch crystals and granules. During the biosynthesis of starch, starch synthase, starch branching enzyme and starch debranching enzyme work together to form the starch granules. In the absence of starch DBE enzyme, highly branched phytoglycogen is formed instead of starch granules. The molecular density of phytoglycogen is over 10 times higher than that of starch. The density of

phytoglycogen extracted from corn is about  $1000 \text{ g/mol}\cdot\text{nm}^3$ , whereas for starch is about  $50 \text{ g/mol}\cdot\text{nm}^3$ . This high density allows for the grafting of different functional groups at its surface and also provides structural integrity.

Chemical modifications are performed to polysaccharides to impart useful functional properties. One such substitution to induce both negative charge and hydrophobicity to phytoglycogen is octenyl succinylation. This modification results in the formation of phytoglycogen octenyl succinate (PG-OS). This substitution has been allowed by the FDA to be used in food systems. The surface properties of PG-OS can be controlled by the degree of substitution (DS) value.

### 1.10 Goals and objectives

Overall goals: To understand the interaction and protection of antimicrobial compounds with carbohydrate based colloidal systems.

#### 1.10.1 Chapter 2

Objectives: To (1) study adsorption process between nisin and PG-OS nanoparticles in non-emulsion or aqueous systems and (2) study adsorption process between nisin and PG-OS stabilized interfacial layer in emulsion systems.

#### 1.10.2 Chapter 3

Objectives: To (1) explore the factor responsible for nisin depletion in cantaloupe juice, (2) study the prolonged efficacy of nisin when combined with starch-OS emulsion as a delivery vehicle in cantaloupe juice model against *Listeria monocytogenes* V7 and (3) understand the underlying mechanism of prolonged nisin efficacy when incorporated with starch-OS emulsion.

#### 1.10.3 Chapter 4

Objectives: To (1) study the prolonged efficacy of nisin and thymol when combined with starch-OS emulsion as a delivery vehicle in cantaloupe juice model against *Listeria monocytogenes* V7 and *Salmonella* Typhimurium, and (2) understand the effect of antimicrobial partitioning on their prolonged efficacy.

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## CHAPTER 2. COMPARATIVE STUDY ON THE INTERACTIONS BETWEEN NISIN AND CARBOHYDRATE NANOPARTICLES IN NON-EMULSION AND EMULSION SYSTEMS

### 2.1 Abstract

Antimicrobial compounds, used extensively in protecting foods against pathogens suffer from degradation because of various depletion factors. One way to achieve prolonged protection of antimicrobials is by combination with a delivery agent. The overall goal of this study was to understand the interaction between antimicrobial peptide, nisin and phytoglycogen octenyl succinate (PG-OS) nanoparticles as delivery vehicle in aqueous (non-emulsion) and emulsion systems. Nisin interacts with PG-OS particles following a Langmuir monolayer adsorption pattern in both systems. The monolayer adsorption capacity ( $Q_m$ ) increased from 37.6 to 106.4  $\mu\text{g/mL}$  in non-emulsion with an increase of degree of substitution (DS) of PG-OS from 0.010 to 0.074. The  $Q_m$  value in the emulsion was 181.8  $\mu\text{g/mL}$ . Importantly, at the same amount of PG-OS (5.0 mg/mL) and nisin (200  $\mu\text{g/mL}$ ), the concentration of nisin adsorbed in aqueous and emulsion systems were 22 and 157  $\mu\text{g/mL}$ , respectively. These results revealed that PG-OS distributed at oil-water interface may substantially increase adsorption of nisin to the colloidal system.

**Keywords:** Phytoglycogen octenyl succinate, nisin, adsorption, emulsion

## 2.2 Introduction

Food borne pathogens and spoilage organisms are a major concern for public health. In 2012, CDC estimated that 1 out of every 6 Americans became sick due to food borne pathogens, including bacteria, parasites and viruses (Scallan, et al., 2011). A number of processing methods are available to keep food safe. These include thermal processing, freezing, high pressure processing, microwave and irradiation treatments (Farkas, 1998; Oey, Lille, Van Loey, & Hendrickx, 2008). Along with conventional food processing methods, alternative approaches are desirable to improve efficacy and reduce cost. Antimicrobial compounds can be a viable option (Tiwari, Valdramidis, O' Donnell, Muthukumarappan, Bourke, & Cullen, 2009). Antimicrobial compounds are found universally within the prokaryotic and eukaryotic world, among which nisin is a widely used peptide (Tiwari, Valdramidis, O' Donnell, Muthukumarappan, Bourke, & Cullen, 2009). Produced from *Lactococcus lactis* (de Arauz, Jozala, Mazzola, & Penna, 2009), nisin is a polycyclic, cationic antibacterial peptide comprising of 34 amino acid residues (Bonev, Chan, Bycroft, Roberts, & Watts, 2000; Breukink, Ganz, de Kruijff, & Seelig, 2000; W. Liu & Hansen, 1990). It has been found to be highly effective against Gram-positive bacteria such as *Listeria monocytogenes*, a major food borne pathogen. Nisin kills bacteria by forming pores on cell membranes (Breukink & de Kruijff, 2006).

However, nisin is highly unstable in food systems and may lose its antibacterial efficacy over a very short period of time. This is mostly due to the diffusion of nisin into the food mass or interactions with other food components such as proteases, glutathione, sodium metabisulphite and titanium dioxide (Bhatti, Veeramachaneni, & Shelef, 2004; Jung, Bodyfelt, & Daeschel, 1992; Mahadeo & Tatini, 1994; Rose, Sporns, Stiles, & McMullen, 1999). Several strategies have been tried to prolong nisin efficacy such as incorporation in packaging films, edible coatings, biopolymeric capsules, and liposomes (Ercolini, Ferrocino, La Stora, Mauriello, Gigli, Masi, et al., 2010; T. Jin, L. Liu, H. Zhang, & K. Hicks, 2009; Laridi, Kheadr, Benez, Vuilleumard, Lacroix, & Fliss, 2003; Schmidt, Holub, Sturino, & Matthew Taylor, 2009; Taylor, Davidson, Bruce, & Weiss,

2005). Nisin has also been incorporated with different types of nanoparticles, such as chitosan-alginate complex (Zohri, Shafiee Alavidjeh, Mirdamadi, Behmadi, Hossaini Nasr, Eshghi Gonbaki, et al., 2013), liposomal nanoparticles (Zou, Lee, Seo, & Ahn, 2012), solid lipid nanoparticles (Prombutara, Kulwatthanasal, Supaka, Sramala, & Chareonpornwattana, 2012) and metallic nanoparticles such as MgO (Jin & He, 2011). Nisin loaded chitosan-alginate complex resulted in five and seven fold  $\log_{10}$  CFU/mL reduction against *Staphylococcus aureus* and *Listeria monocytogenes*, respectively in ultrafiltered Feta cheese (Zohri, et al., 2013). In BHI agar medium, Zou et al., 2012, found that nanoliposome encapsulated nisin could generate 6  $\log_{10}$  CFU/mL reduction of *Staphylococcus* and *Listeria*. Nisin combined with solid lipid nanoparticles (SLN) could demonstrate antimicrobial efficacy against *Listeria monocytogenes* DMST 2871 and *Lactobacillus plantarum* TISTR 850 for 15 days, when free nisin was depleted in 3 days (Prombutara, Kulwatthanasal, Supaka, Sramala, & Chareonpornwattana, 2012).

Research in our laboratory has shown that nisin can be protected for a prolonged period of time by combining with phytoglycogen octenyl succinate (PG-OS) nanoparticles in both aqueous (non-emulsion) and emulsion based systems (Bi, Yang, Bhunia, & Yao, 2011a; Bi, Yang, Narsimhan, Bhunia, & Yao, 2011). Using a model depletion system, the PG-OS dispersion retained nisin activity for up to 21 days in comparison with 7 days for nisin in aqueous solution. In contrast, PG-OS emulsion retained nisin activity for up to 50 days in comparison with 15 days for nisin alone preparation. In the present work, our goal was to study nisin adsorption with PG-OS in both non-emulsion and emulsion systems. To achieve this, phytoglycogen (PG) was extracted from mutant corn and modified using various levels of octenyl succinate (OS) to generate PG-OS with different degrees of substitution. The PG-OS materials were dispersed in the form of non-emulsion and emulsion, and their nisin adsorption capabilities were compared.

## 2.3 Materials and Methods

### 2.3.1 Materials

The sweet corn used for phytoglycogen isolation was obtained commercially from an industrial company. Octenyl succinic anhydride was provided by Dixie Chemical Co. (Houston, TX). Dialysis membranes (molecular weight cut-off of 300 kDa) and Fast Micro-Equilibrium Dialyzer unit (FMED) were purchased from Harvard Apparatus (Holliston, MA). Nisin was obtained from Silver Elephant Company (Zhejiang, China) and analyzed using HPLC for purity.

### 2.3.2 Phytoglycogen extraction

Phytoglycogen (PG) was extracted from sweet corn kernels. The kernels of sweet corn (300 g) were firmly ground using a commercial blender (Waring Laboratory, Torrington, CT) and mixed with ten times of deionized water on a w/w basis. This mix was further blended using a high-speed blender (Waring Laboratory, Torrington, CT). The slurry was then centrifuged at  $10,000 \times g$  for 20 min. The supernatant was passed through a 270-mesh sieve. Ethanol (3 volumes) was added to the clear liquid to precipitate the phytoglycogen (PG). This was centrifuged again at  $10,000 \times g$  for 20 min and the supernatant decanted. Ethanol was used to suspend the precipitate and vacuum filtered three times to dry the material. The powder after vacuum filtration was air dried overnight to obtain phytoglycogen (PG) (Scheffler, Wang, Huang, San-Martin Gonzalez, & Yao, 2009).

### 2.3.3 Phytoglycogen octenyl succinate preparation

To prepare phytoglycogen octenyl succinate (PG-OS), phytoglycogen (PG) was reacted with octenyl succinic anhydride at different ratios (3 - 15% w/w based on PG). For substitution, 20% w/w native phytoglycogen was dispersed in distilled water and octenyl succinic anhydride (OS) was added in 2 h at four different levels: 3%, 7%, 10% and 15%, based on the dry weight of glucan. The pH was maintained between 8.5 to 9.0

by using 0.5 (M) NaOH solution and the reaction temperature was maintained at 40 °C. The reaction was terminated after 24 h by pH reduction to 6.5 with the addition of 0.5 (M) HCl. Then the neutralized reaction mixture was added to three volumes of ethanol and dehydrated using 3 cycles of ethanol suspension and centrifugation. The material collected was kept inside the fume hood for 24 h to obtain dried PG-OS powder (Scheffler, Huang, Bi, & Yao, 2010; Scheffler, Wang, Huang, San-Martin Gonzalez, & Yao, 2009).

#### 2.3.4 Phytoglycogen octenyl succinate: structural analysis

Weight-average molar mass ( $M_w$ ) and root mean square radius ( $R_z$ ) of PG-OS particulates were determined using high performance size exclusion chromatography connected with multi angle laser light scattering and refractive index detectors (HPSEC-MALLS-RI) (MALLS and RI from Wyatt Technology, Santa Barbara, CA) as described by Huang & Yao (2011). Two connected columns (PL Aquagel-OH 40 and 60) were used with a guard column (Polymer Laboratories, Varian Inc.). Molecular density was calculated from  $M_w$  and  $R_z$  using equation  $\rho = \frac{M_w}{R_z^3} \text{ g/mol} \cdot \text{nm}^3$  (Huang & Yao, 2011; Scheffler, Huang, Bi, & Yao, 2010).

#### 2.3.5 Phytoglycogen octenyl succinate: zeta-potential measurement

Zetasizer Nano (ZS90, Malvern, Worcestershire, UK) was used to measure the zeta-potential of PG-OS particulates. For the measurement, PG-OS was dispersed in sodium acetate buffer (50 mM, pH 5.5) to around 0.10 µg/mL. The measurement was performed at 25 °C under an automatic setting condition with 1 min equilibration time.

#### 2.3.6 Transmission electron microscopy of phytoglycogen octenyl succinate (PG-OS)

Transmission electron microscopy (TEM) of native PG and various PG-OS materials was performed according to the procedure of (Scheffler, Huang, Bi, & Yao, 2010). For specimen preparation, the concentration of PG-OS materials in sodium acetate buffer (20 mM, pH 5.5) was 0.05% (w/v).



### 2.3.7 Preparation of nisin standard curve

Highly enriched nisin solid was obtained from Silver Elephant Company (Zhejiang, China). This material contained approximately 77% of nisin-A based on our HPLC analysis (data not shown). Based on this information, we used the protein amount determined using Bradford assay as an approximate value of nisin peptide, well realizing that this may lead to certain errors in the calculation of nisin amount (discussed later in the result section). For Bradford assay, solution of nisin at 1.0 mg/mL in sodium acetate buffer (50 mM, pH 5.5) was diluted to make standard solutions ranging from 2.0 to 20.0  $\mu\text{g/mL}$ . The nisin content was determined using a UV-vis spectrophotometer (DU 730 Beckman Coulter, CA) at 595 nm.

### 2.3.8 Incorporation of nisin with phytylglycogen octenyl succinate (PG-OS) in non-emulsion

Nisin powder was dissolved in sodium acetate buffer (50 mM, pH 5.5) to form a 1.0 mg/mL stock solution based on the purity of nisin. Each of PG-OS materials was dissolved in sodium acetate buffer (50 mM, pH 5.5) at 10.0 mg/mL. Thereafter, the nisin and PG-OS solutions were combined in tubes at an equal volume and agitated at 70 rpm on a rotary shaker (Lab-Line, Max/Rotator, USA) overnight at room temperature (22 °C). The mixtures of nisin and PG-OS solutions were further subjected to equilibrium dialysis.

### 2.3.9 PG-OS stabilized emulsion preparation

To prepare PG-OS stabilized emulsion, PG-OS with DS of 0.010 was dissolved in sodium acetate buffer (50 mM, pH 5.5) at 5.0 mg/mL. To this solution, soybean oil was added to achieve 2.50 mg/mL. The mixture was vortexed and subjected to a high-speed homogenizer (Ultra Turrax, IKA T25, NC) at 18,000 rpm for 1 min and then a high-pressure homogenizer (GEA Panda Plus, Italy) at 10,000 psi for 3 cycles. To the emulsion generated, nisin material was added to achieve concentrations ranging from 10 to 500  $\mu\text{g/mL}$ , and the mixture was agitated on a rotary shaker (Lab-Line Max-Rotator) at 70 rpm overnight at room temperature (22 °C). The emulsions were characterized for their zeta-potential and particle size distribution using Zetasizer (ZS90, Malvern,

Worcestershire, UK). For both the measurements, the emulsions were diluted 100 times in volume using sodium acetate buffer (50 mM, pH 5.5). The mixtures of nisin and PG-OS stabilized emulsions were further subjected to equilibrium dialysis.

#### 2.3.10 Estimation of nisin adsorption: equilibrium dialysis experiments

Equilibrium dialysis was employed to determine nisin adsorption with PG-OS particulates in non-emulsion (aqueous) and emulsion systems. Fast micro-equilibrium dialyzers (FMED, Harvard Apparatus, MA) were used to determine the concentration of non-adsorbed nisin. The FMED unit consists of 2 dialysis chambers separated by a membrane, each with a volume of 500  $\mu$ L. For dialysis, 100 mL of fluid that contained PG-OS and nisin was added in a glass vial. A cellulose acetate dialysis membrane with a molecular weight cut-off (MWCO) of 300 kDa was inserted in the dialysis unit to form permeate compartment. Thereafter, the unit was submerged in the fluid in the vial for dialyzing operation. The volume of permeate compartment was 500  $\mu$ L, which was nearly negligible compared with the total volume of dialyzing fluid (100 mL). Therefore, the use of dialyzer was considered as nearly non-invasive to the partitioning of nisin in the bulk of fluid.

Figure 2.1a and 2.1b provide schematics of the equilibrium dialysis for non-emulsion and emulsion based colloidal systems, respectively. For the operation, the permeate compartment of each dialyzer was filled with 500  $\mu$ L sodium acetate buffer (50 mM, pH 5.5) and placed in a vial that contained the mixture of nisin and PG-OS (either non-emulsion or emulsion). The vial was sealed and allowed to be agitated on a horizontal water-bath (VWR, USA) ) at 70 rpm and 15 °C for 24 h. Preliminary tests using 200  $\mu$ g/mL nisin showed that 24 h was sufficient for reaching equilibrium. For the estimation of the equilibrium time, nisin in aqueous solution at 200  $\mu$ g/mL was allowed to be agitated in a similar set-up as described above. Fluids were collected after 6, 9, 12, 15, 18, 21 and 24 h and quantified for nisin concentration using Bradford Microassay technique. Bradford Microassay procedure was performed by using the dye reagent of Quick Start Bradford Protein Assay kit. After incubation at room temperature for 10 min, the absorbance of the solutions were measured using a UV-Vis spectrophotometer (DU 730 Beckman Coulter,

CA) at 595 nm. For the actual samples, solutions were withdrawn from the permeate compartment after 24 h of dialysis and subjected to Bradford assay with the standard curve earlier described.

#### 2.3.11 Interaction between nisin and PG-OS: Langmuir monolayer adsorption

The Langmuir monolayer adsorption model was used to determine the adsorption behavior of nisin with PG-OS particles in non-emulsion and emulsion systems. This model can be expressed as  $Q_e = \frac{Q_m K C_e}{1 + K C_e}$ , where  $Q_m$  is the monolayer adsorption capacity,  $C_e$  the concentration of non-adsorbed nisin at equilibrium,  $Q_e$  is the concentration of adsorbed nisin at equilibrium, and  $K$  is the equilibrium constant. The Langmuir equation can be transformed to  $\frac{1}{Q_e} = \frac{1}{Q_m} + \frac{1}{Q_m K C_e}$  that can be used to determine  $Q_m$  and  $K$  (Foo & Hameed, 2010).

#### 2.3.12 Statistical analysis

The data, when appropriate, are expressed as mean  $\pm$  SD with sample size (n) of 3. ANOVA analysis was performed ( $p < 0.05$ ) using SPSS software (version 21, IBM, USA). Tukey's HSD multiple comparison test was performed to evaluate the difference among treatments.

### 2.4 Results

#### 2.4.1 Structural characterization of phytoglycogen octenyl succinate (PG-OS)

The physical and structural parameters of 4 types of PG-OS particulates are shown in Table 2.1. The degree of substitution (DS) of PG-OS increased with an increased use of octenyl succinate (OS). The DS values for 3, 7, 10 and 15% OS usage were 0.010, 0.026, 0.049 and 0.074, respectively. The substitution efficiency (SE) (SE = measured DS/theoretical DS with 100% yield) for PG-OS of DS 0.010, 0.026, 0.049 and 0.074 were 42%, 48%, 63% and 61%, respectively. The weight-average molar mass of the particles showed an increasing trend from  $1.59$  to  $1.91 \times 10^7$  as the DS value

increased from 0 to 0.026. With further increase of DS, however, the weight average molar mass of PG-OS did not change significantly. The root-mean square radius ( $R_z$ ) values increased from 24.3 nm to 30.1 nm when the DS value increased from 0 to 0.074. Such an increase is possibly due to an increased electrostatic repulsion among negatively charged moieties of octenyl succinate (OS) on the surface of the PG-OS particulates. As a result, the molecular density decreased from 1099 to 689 g/mol·nm<sup>3</sup> with an increase of DS from 0.010 to 0.074. Similar trend in the increase of root mean square radius and decrease in the molecular density of PG-OS resulted from an increase of DS were also observed by (Scheffler, Huang, Bi, & Yao, 2010; Scheffler, Wang, Huang, San-Martin Gonzalez, & Yao, 2009).

#### 2.4.2 TEM imaging of phytoglycogen and its derivatives

The transmission electron microscopy (TEM) imaging of native and modified PG particulates revealed spheroidal structure, consistent with previous reports (Bi, Yang, Narsimhan, Bhunia, & Yao, 2011; Huang & Yao, 2011; Putaux, Buleon, Borsali, & Chanzy, 1999). As shown in Figure 2.2, PG particles ranged between 30 to 100 nm. TEM images showed that an increased DS of PG-OS scattered the individual PG-OS particles, suggesting the role of increased electrostatic repulsion.

#### 2.4.3 Zeta-potentials of PG-OS materials in non-emulsion

Zeta-potential is defined as the electrostatic potential between the plane of shear (within the interfacial double layer) and the bulk fluid away from the interface. Zeta-potential is an important parameter that describes the interactions among charged groups and the stability of colloidal systems. In this study, zeta-potential was used to reveal the electrostatic interactions between positively charged nisin molecules and negatively charged PG-OS particles. As shown in Figure 2.3, the DS value of octenyl succinate groups substantially affects the zeta-potential of PG-OS particulates. The zeta-potential of native PG was -3.09 mV (Table 2.1), possibly related to the residual amount of protein (Bi, Yang, Narsimhan, Bhunia, & Yao, 2011). With increasing DS, the mean zeta-potential values were -15.2, -19.4, -23.8 and -26.5 mV for PG-OS with DS of 0.010,

0.026, 0.049 and 0.074, respectively. The addition of nisin decreased the absolute value of zeta-potential of PG-OS particulates, and such a decrement was affected by the DS value. Adding 10 and 200  $\mu\text{g/mL}$  of nisin to PG-OS of DS 0.010, 0.026, 0.049 and 0.074, the absolute zeta-potential values reduced to -10.7, -13.1, -17.4, -21.9 mV and -7.7, -8.2, -12.2, -14.1 mV, respectively.

#### 2.4.4 PG-OS emulsion: zeta-potential and particle size distribution

Zeta-potential was also used to describe the electrostatic interactions between nisin molecules and the oil droplets with PG-OS of DS 0.010 stabilized emulsion. The zeta-potential values of emulsion stabilized by PG-OS with DS of 0.010 with and without added nisin are shown in Figure 2.3. With nisin added at 0, 10, 50, 200, and 500  $\mu\text{g/mL}$ , the zeta-potential values changed to -15.7, -13.4, -8.9, -7.6, and -3.6 mV, respectively. This result was consistent with our previous studies (Bi, Yang, Bhunia, & Yao, 2011), in which the zeta-potential of PG-OS with DS of 0.013 emulsion was -15.5 mV and when mixed with nisin 150 and 200  $\mu\text{g/mL}$  the zeta-potential changed to -9 and -5 mV, respectively.

The particle size distribution of PG-OS with DS of 0.010 emulsion is shown in Figure 2.4. The droplet size (or the hydrodynamic diameter,  $D_H$ ) of PG-OS with DS of 0.010 emulsion, without added nisin, was about 359.4 nm.

#### 2.4.5 Nisin adsorption to PG-OS in aqueous system

Figure 2.5 shows the estimation of equilibrium time for nisin using an equilibrium dialysis set-up. The internal dialysis chamber equilibrated with the outside nisin concentration (200  $\mu\text{g/mL}$ ) at around 24 h of interaction. Based on this data, we used 24 h for all the subsequent experiments. Figure 2.6 shows the amount of adsorbed nisin versus total nisin concentrations in non-emulsion (aqueous) system. For example, at 10 and 200  $\mu\text{g/mL}$ , the adsorbed nisin concentrations corresponding to DS of 0.010, 0.026, 0.049 and 0.074 were 2.9, 3.2, 3.5, 4.7  $\mu\text{g/mL}$  and 21.5, 28.1, 38.8 and 49.2  $\mu\text{g/mL}$ , respectively. The linearized forms of Langmuir adsorption isotherms are shown in Figure 2.7. From the linearized Langmuir adsorption plots, the monolayer adsorption capacity

( $Q_m$ ) values were calculated as 37.6, 58.8, 87.7 and 106.4  $\mu\text{g/mL}$ , respectively for PG-OS with DS of 0.010, 0.026, 0.049 and 0.074 (Table 2.2). On the other hand, the adsorption equilibrium constant ( $K$ ) was only slightly affected by the increase of OS groups: with DS of 0.010, 0.026, 0.049 and 0.074, the  $K$  value was 11.6, 8.3, 7.0 and  $9.5 \times 10^{-3}$   $\text{mL}/\mu\text{g}$ , respectively. (Table 2.2).

#### 2.4.6 Nisin adsorption in emulsion stabilized with PG-OS of DS 0.010 material

Equilibrium dialysis was also used to evaluate the adsorption of nisin in emulsion. Figure 2.8 shows the nisin adsorption behavior in the emulsion stabilized using PG-OS with DS of 0.010. When total nisin concentrations were 10, 100, 200, 300, 400, and 500  $\mu\text{g/mL}$ , the concentration of adsorbed nisin were 5.6, 32.5, 157, 193, 229, and 241  $\mu\text{g/mL}$ , respectively. The  $Q_m$  value was calculated to be 181.8  $\mu\text{g/mL}$  from linearized form of the Langmuir monolayer isotherm (Table 2, Figure 2.9). The equilibrium constant value ( $K$ ) was  $6.9 \times 10^{-3}$   $\text{mL}/\mu\text{g}$ .

Nisin adsorption behavior showed substantial difference in non-emulsion and emulsion systems. For example, at 200  $\mu\text{g/mL}$  of total nisin, the concentration of adsorbed nisin in the non-emulsion and emulsion was 22 and 157  $\mu\text{g/mL}$ , respectively (Figure 2.10). Undoubtedly, with PG-OS as a nisin delivery vehicle, an emulsion is much more effective in adsorbing nisin than a non-emulsion, aqueous only colloidal system. The effectiveness of emulsion system to prolong nisin activity was revealed in our previous work. In that study, emulsion formed using PG-OS with DS of 0.013, when loaded with 150 or 200  $\mu\text{g/mL}$  nisin, could prolong the anti-listerial activity of nisin from 10 to 50 days (Bi, Yang, Bhunia, & Yao, 2011a).

## 2.5 Discussion

This study was an attempt to understand the interactions between negatively charged PG-OS nanoparticles and a positively charged peptide, nisin in different types of dispersion systems. The interactions between particles or surfaces and active compounds can be studied by understanding their adsorption behavior. Adsorption isotherm is a

curve that shows the retention, release or mobility of an active compound in an environment to a solid-phase at constant conditions of pH and temperature (Foo & Hameed, 2010; Qi & Xu, 2004). The adsorption equilibrium, one of the most important parameter in the isotherm is found by calculating the ratio between the adsorbed concentration to the remaining solution concentration of the active compound (Du, Xu, Han, Xu, & Miao, 2008; Foo & Hameed, 2010; Langmuir, 1916).

The use of Langmuir equation would allow us to better understand the interactions among nisin molecules and PG-OS particulates in simple dispersion and emulsion systems. The Langmuir equation assumes that: (1) the adsorption takes place at a fixed number of localized sites; (2) there is no steric hindrance between adjacent adsorbate molecules; (3) each adsorption site can bind only one molecule; (4) the sites are homogeneous in nature and (5) adsorption surface have equivalent sorption energies (Foo & Hameed, 2010; Langmuir, 1916; Vijayaraghavan, Padmesh, Palanivelu, & Velan, 2006). The data with both non-emulsion and emulsion systems demonstrated that Langmuir monolayer equation can be used to describe nisin adsorption phenomenon. In both systems, PG-OS particulates were the adsorbent and nisin was the adsorbate. In the non-emulsion, the increase in the monolayer adsorption capacity ( $Q_m$ ) value with increase in the DS of PG-OS particles is due to higher concentration of negatively charged carboxylate groups on the PG-OS surface. Due to higher negative charge density, there is higher level of binding between the negatively charged carboxylate groups and positively charged nisin molecules. Therefore, at the surface of PG-OS particulates, nisin adsorption leads to the formation of a monolayer. After monolayer formation, this single layer of nisin molecules possibly repels the adsorption of additional nisin molecules.

The adsorption of nisin to PG-OS stabilized emulsion needs further discussion. In this study, the total amount of nisin and PG-OS materials were the same in the non-emulsion and emulsion systems. In addition, other adsorption conditions such as pH and temperature were also constant. It seems reasonable to suggest that PG-OS structural feature could be the driving force behind enhanced nisin adsorption. Figure. 2.11 provides a schematic comparison between a simple dispersion and emulsion. This well-defined PG-OS colloidal assembly at the droplet surface could lead to two important

phenomena: (1) a collaborative effect among adjacent PG-OS materials can enhance nisin adsorption due to both electrostatic and hydrophobic interactions and (2) nisin can also interact with oil droplet surface due to hydrophobic interactions, which may contribute to the total binding sites for nisin adsorption.

In addition, the adsorption of nisin to PG-OS surface and PG-OS stabilized interface can be explained by the use of the Clausius-Clapeyron equation. The Langmuir adsorption isotherm is mathematically defined as  $Q_e = \frac{Q_m K C_e}{1 + K C_e}$ . K is the equilibrium constant for the interaction between adsorbate and adsorbent. This K value is actually a ratio between the rate constant of adsorption ( $k_a$ ) to the rate constant for desorption ( $k_d$ ), therefore, mathematically  $K = \frac{k_a}{k_d}$ . The product ( $Q_m K$ ) in the Langmuir equation numerator is connected to the Clausius-Clapeyron equation in the following manner:  $Q_m K = A \exp(-\frac{\Delta H}{RT})$ , where,  $\Delta H$  is the change in adsorption enthalpy, R is the universal gas constant, T is the absolute temperature and A is a constant. Taking  $\log_e$  function on both sides and rearranging, the equation can be transformed to  $(\ln A - \ln Q_m K)RT = \Delta H$ .

From our non-emulsion (aqueous) and emulsion data, the  $[Q_m K]$  values were calculated. The  $Q_m K$  value for aqueous system was 0.44 and for emulsion was 1.26. Since the product of  $Q_m$  and K for emulsion is higher compared to non-emulsion, the  $\Delta H$  value is lower in emulsions compared to non-emulsions. A lower  $\Delta H$  value indicates that nisin adsorption to PG-OS interfacial layer is thermodynamically favorable process. Therefore, the PG-OS interfacial region of emulsion is more stable for nisin adsorption. In addition to the PG-OS, the oil droplets also provide significant amounts of weak hydrophobic binding sites. Nisin contains some hydrophobic amino acids due to which it can possibly interact with the oil droplet surface by weak hydrophobic interactions. Overall, this combined process enhances nisin adsorption to the emulsion droplets, thereby showing a high  $Q_m$  value and a low  $\Delta H$ .



## 2.6 Conclusion

This report addresses the driving forces that govern the adsorption of nisin with PG-OS based colloidal assemblies. In the non-emulsion, degree of substitution (DS) of PG-OS particles can be used to control nisin adsorption. Nisin adsorption to PG-OS in non-emulsion and to PG-OS stabilized emulsion follows a Langmuir monolayer adsorption pattern. For PG-OS with DS of 0.010, 0.026, 0.049 and 0.074, the monolayer adsorption capacity ( $Q_m$ ) values were 37.6, 58.8, 87.7 and 106.4  $\mu\text{g/mL}$ , respectively in the non-emulsion system. The monolayer adsorption capacity ( $Q_m$ ) for PG-OS with DS of 0.010 stabilized emulsion was 181.8  $\mu\text{g/mL}$ . Additionally we demonstrated at the similar concentration of PG-OS with DS of 0.010 (5.0 mg/mL) and total nisin (200  $\mu\text{g/mL}$ ), the adsorbed nisin concentration for non-emulsion and emulsion were 22 and 157  $\mu\text{g/mL}$ , respectively. Overall, this research demonstrates the underlying mechanism of peptide adsorption to dendrimer-like polysaccharide based nanoparticles in non-colloidal and colloidal systems.

## 2.7 References

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Table 2.1 Weight average molar mass (MW), root-mean square radius (Rz), molecular density ( $\rho$ ), degree of substitution (DS), and zeta-potential ( $\zeta$ ) of phytoglycogen octenyl succinate (PG-OS) materials

OS dose for PG modification	M <sub>w</sub>	R <sub>z</sub>	$\rho$	DS	Zeta-potential
% w/w PG basis	g/mol $\times 10^{-7}$	nm	g/mol $\cdot$ nm <sup>3</sup>		mV
0	1.58 $\pm$ 0.005 a	24.3 $\pm$ 0.29 a	1099 $\pm$ 36 a	-	-3.0 $\pm$ 0.10 a
3	1.86 $\pm$ 0.026 b	26.4 $\pm$ 0.31 b	1006 $\pm$ 27 b	0.010 $\pm$ 0.002 a	-15.2 $\pm$ 1.1 b
7	1.92 $\pm$ 0.004 b	26.9 $\pm$ 0.17 b	983 $\pm$ 17 c	0.026 $\pm$ 0.004 b	-19.8 $\pm$ 1.7 c
10	1.85 $\pm$ 0.002 b	28.2 $\pm$ 0.30 c	824 $\pm$ 26 c	0.049 $\pm$ 0.002 c	-23.9 $\pm$ 1.2 d
15	1.89 $\pm$ 0.048 b	30.1 $\pm$ 0.25 d	689 $\pm$ 27 d	0.074 $\pm$ 0.006 d	-26.5 $\pm$ 0.01 d

Data expressed as mean  $\pm$  SD (n=3). Significant differences in each column are denoted with different letters (p<0.05).

Table 2.2 Monolayer adsorption capacity ( $Q_m$ ) and equilibrium constant ( $K$ ) of nisin with phytoglycogen octenyl succinate (PG-OS) based non-emulsion and emulsion systems

Type (DS of PG-OS)	$Q_m$ ( $\mu\text{g/mL}$ )	$K \times 10^3$ ( $\text{mL}/\mu\text{g}$ )	$R^2$
0.010	37.6	11.6	0.990
0.026	58.8	8.3	0.977
0.049	87.7	7.0	0.981
0.074	106.4	9.5	0.960
0.010-Emulsion	181.8	6.9	0.940

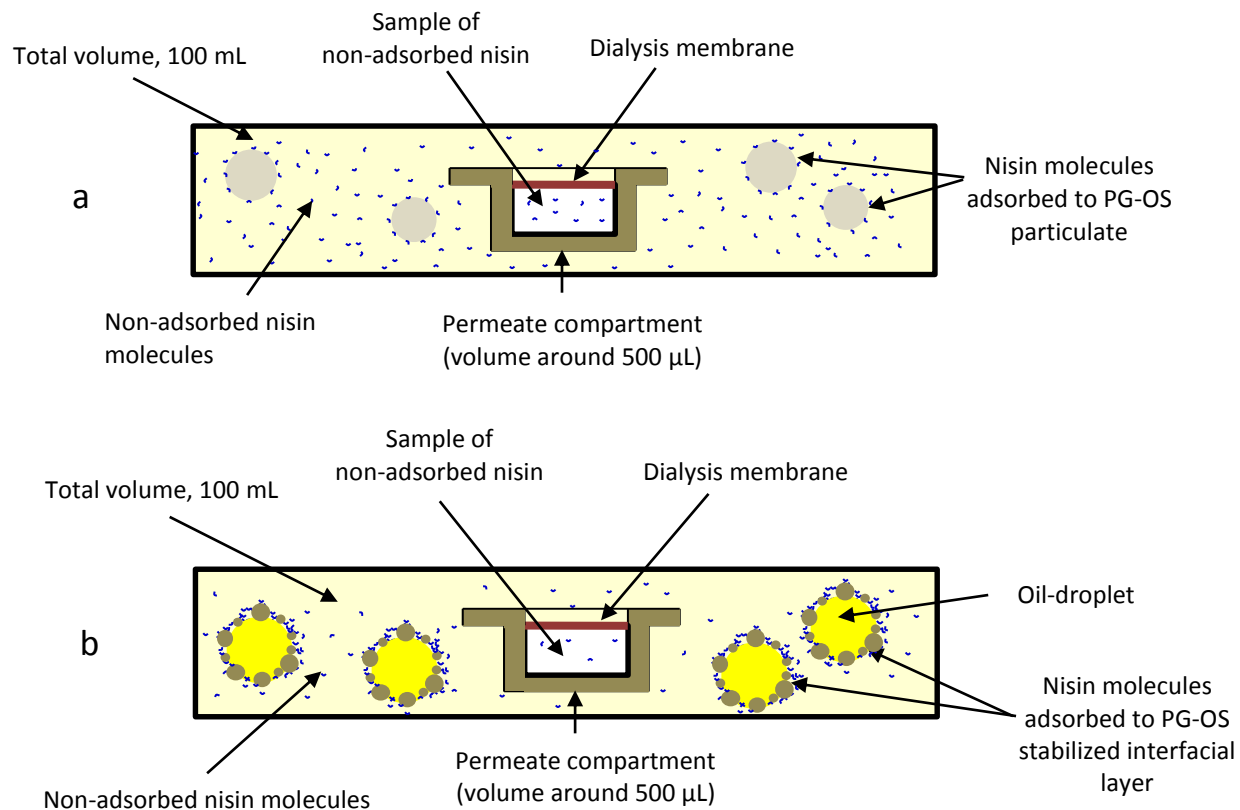
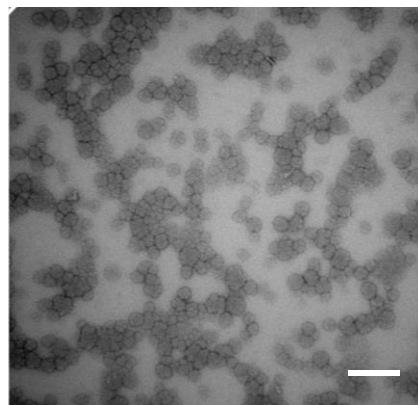
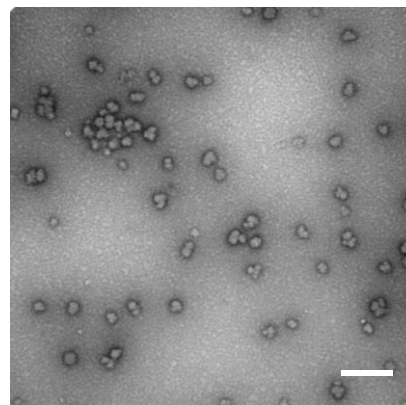


Figure 2.1 Schematic of equilibrium dialysis to quantify non-adsorbed nisin in the non-emulsion (2.1a) and emulsion (2.1b) systems. The PG-OS particles were allowed to interact with nisin molecules; the non-adsorbed nisin was sampled from the permeate compartment of dialysis cell for nisin quantification. The volume of the dialysis permeate compartment was around 500  $\mu$ L, and the nisin samplings were considered nearly non-invasive to the partitioning of nisin molecules in the bulk of fluid (100 mL).

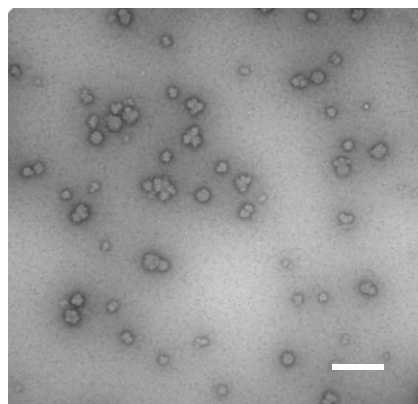
Native PG



0.010



0.049



0.074

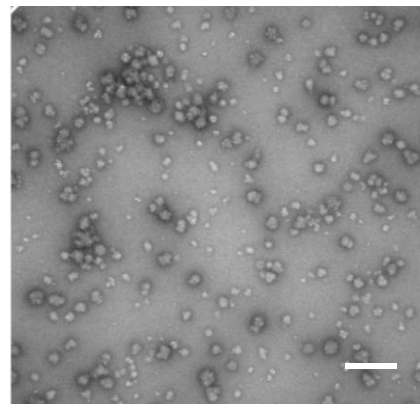


Figure 2.2 Transmission electron microscopy images of phytoglycogen (PG) materials with the degree of substitution (DS) of octenyl succinate of: (a) native PG, (b) 0.010, (c) 0.049, and (d) 0.074. The scale bar shows 200 nm.



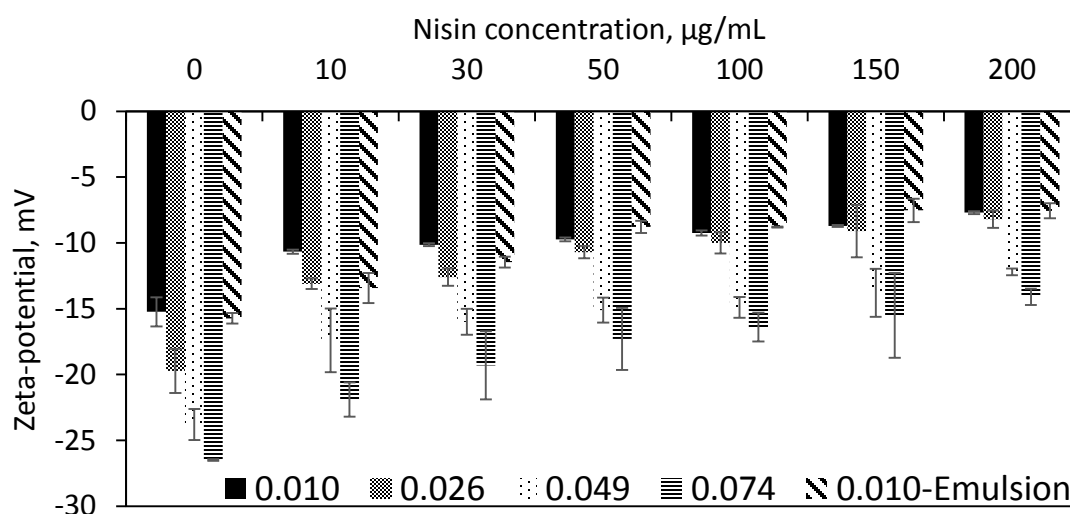


Figure 2.3 Zeta-potential of phytoglycogen octenyl succinate (PG-OS) particulates (0.1 mg/mL, in 10 mM, 5.5 pH sodium acetate buffer) affected by nisin concentration in non-emulsion and emulsion systems. Values of the degree of substitution (DS) of PG-OS are indicated. For each data point (mean value), standard deviations are shown as error bars (n=3).

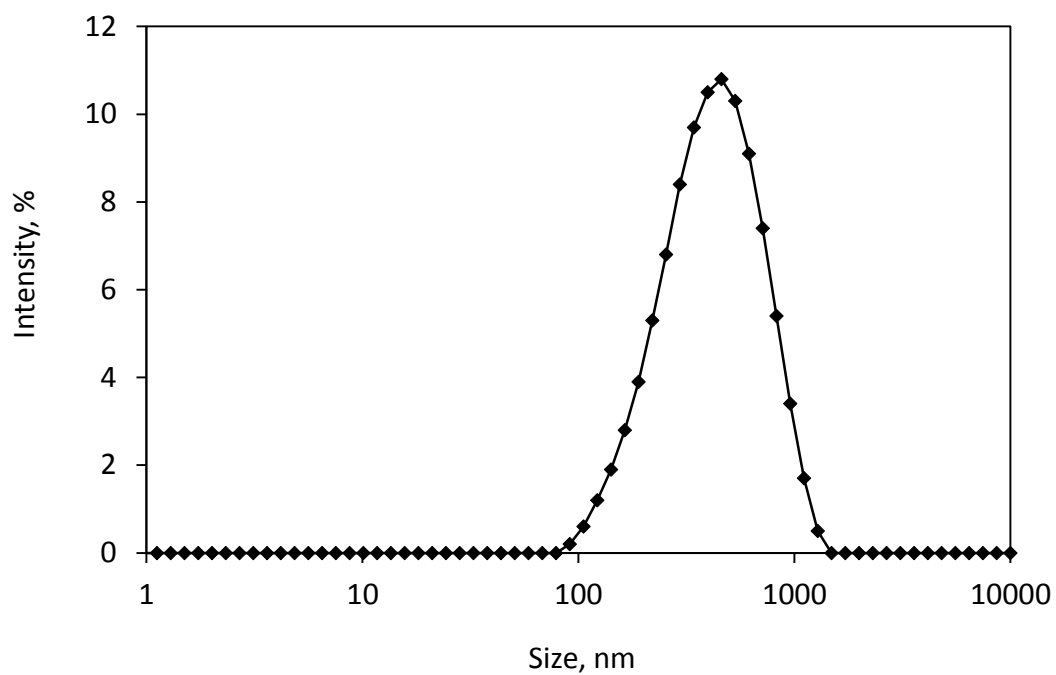


Figure 2.4 Light-scattering intensity-based distribution of hydrodynamic diameter of PG-OS with DS of 0.010 stabilized emulsion droplets without nisin addition.

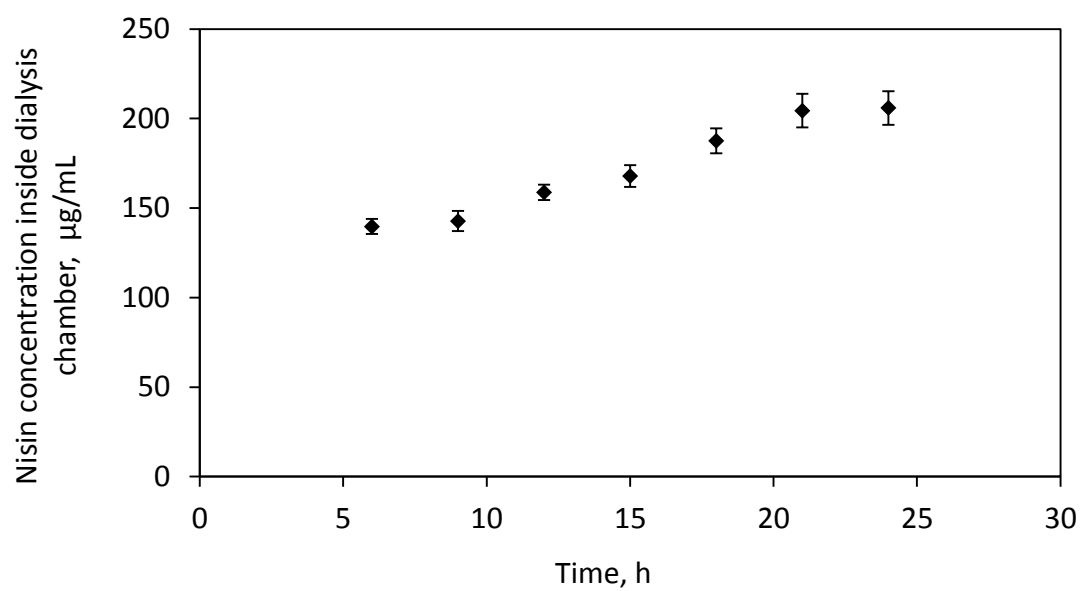


Figure 2.5 Equilibrium time estimation for nisin to reach the outside dialysis chamber concentration (200 µg/mL). Mean values with error bars as standard deviation are shown (n=3).

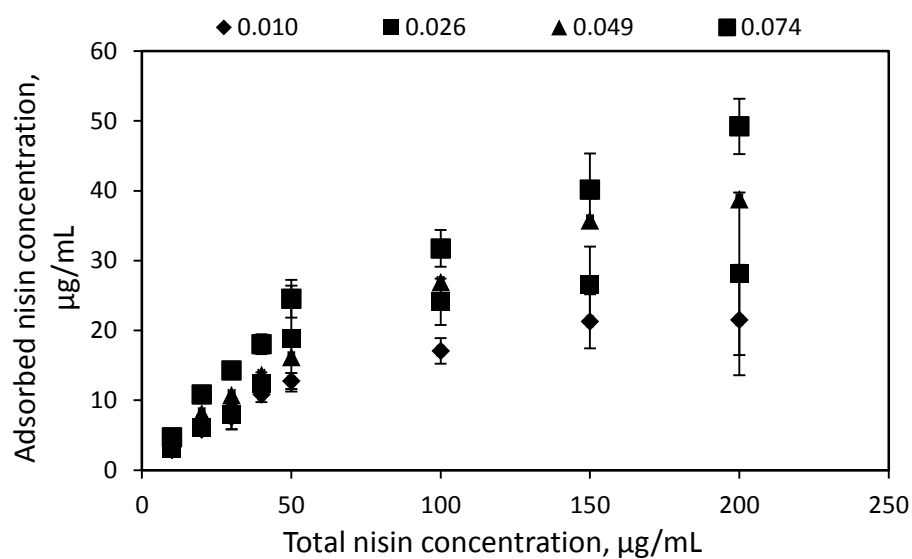
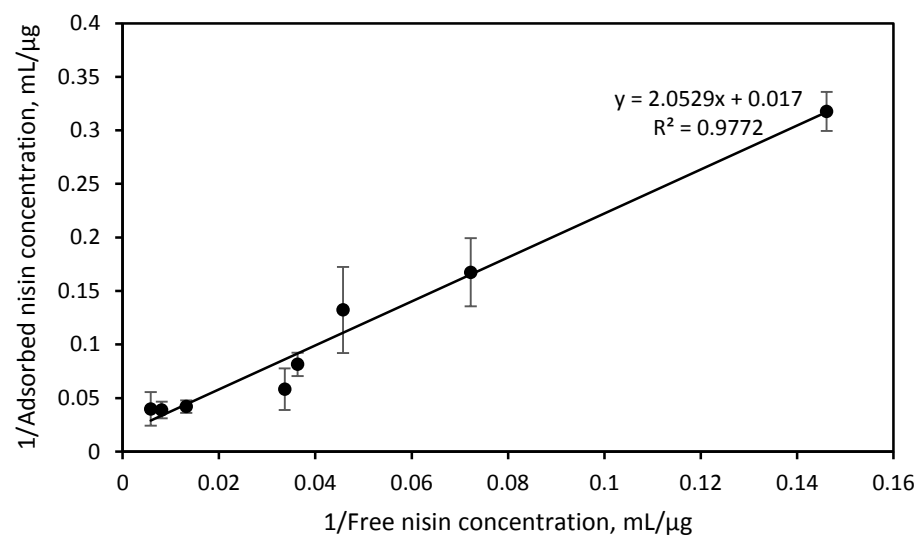
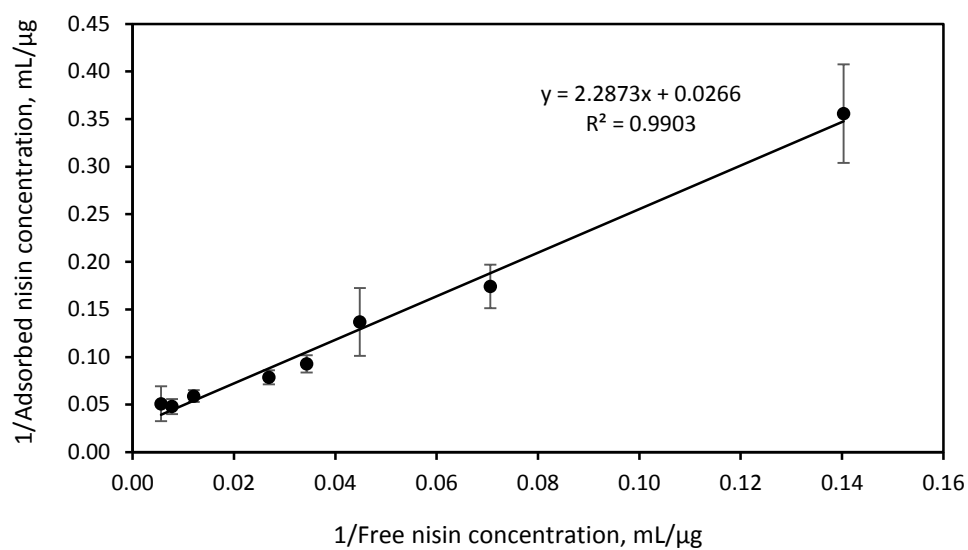


Figure 2.6 Adsorption plot of nisin to each of the four PG-OS particulates with different degree of substitution (DS) in non-emulsion (aqueous) system with nisin concentrations ranging from 10 to 200 µg/mL. Mean values with error bars as standard deviation are shown (n=3).



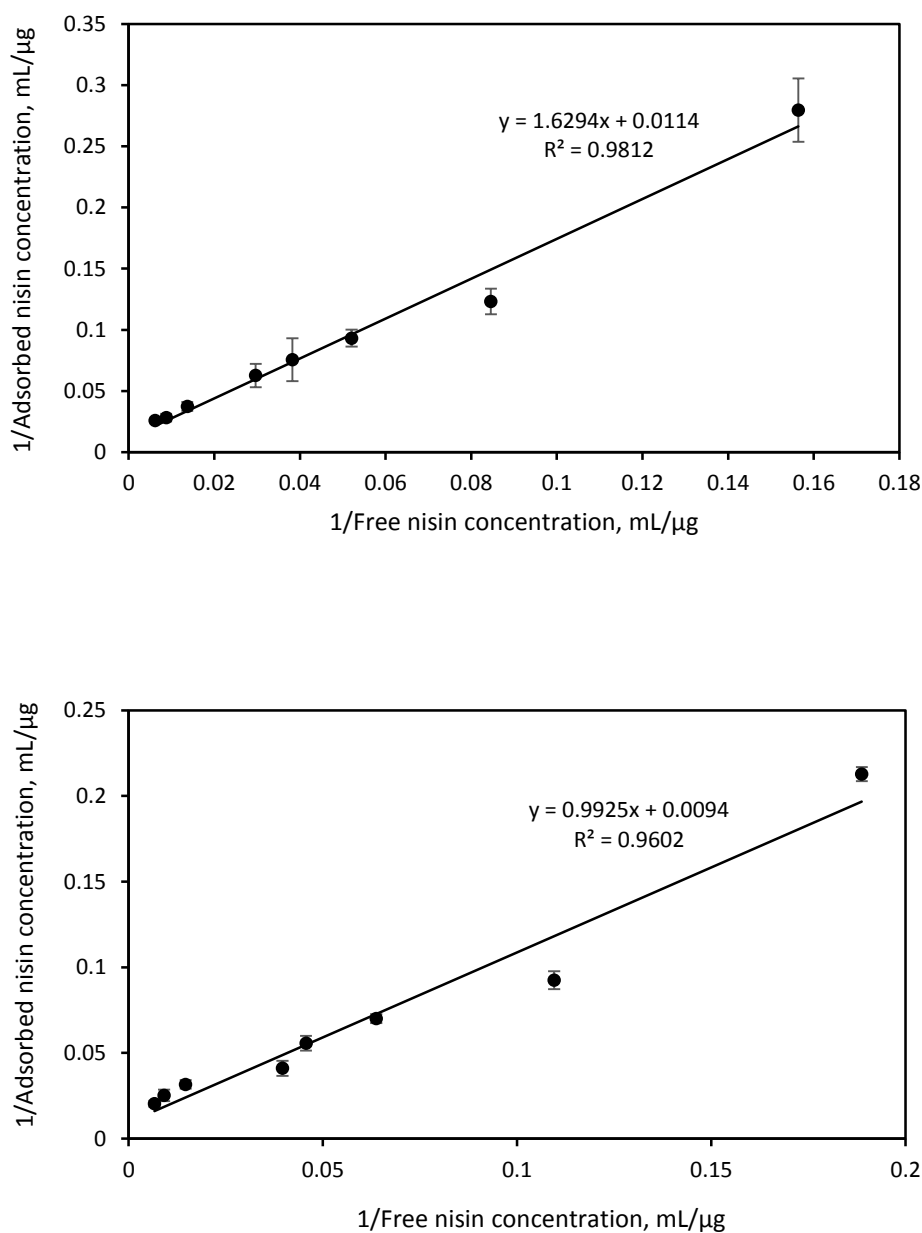


Figure 2.7 Linearized plots of (1/Average adsorbed nisin concentration) versus (1/Average free nisin concentration) to obtain monolayer adsorption capacity ( $Q_m$ ) and equilibrium constant values ( $K$ ) for PG-OS with DS of (A) 0.010, (B) 0.026, (C) 0.049, and (D) 0.074 non-emulsion (aqueous)-nisin samples. Nisin concentrations ranged between 10 to 200  $\mu$ g/mL. Mean values with error bars as standard deviation are shown (n=3).

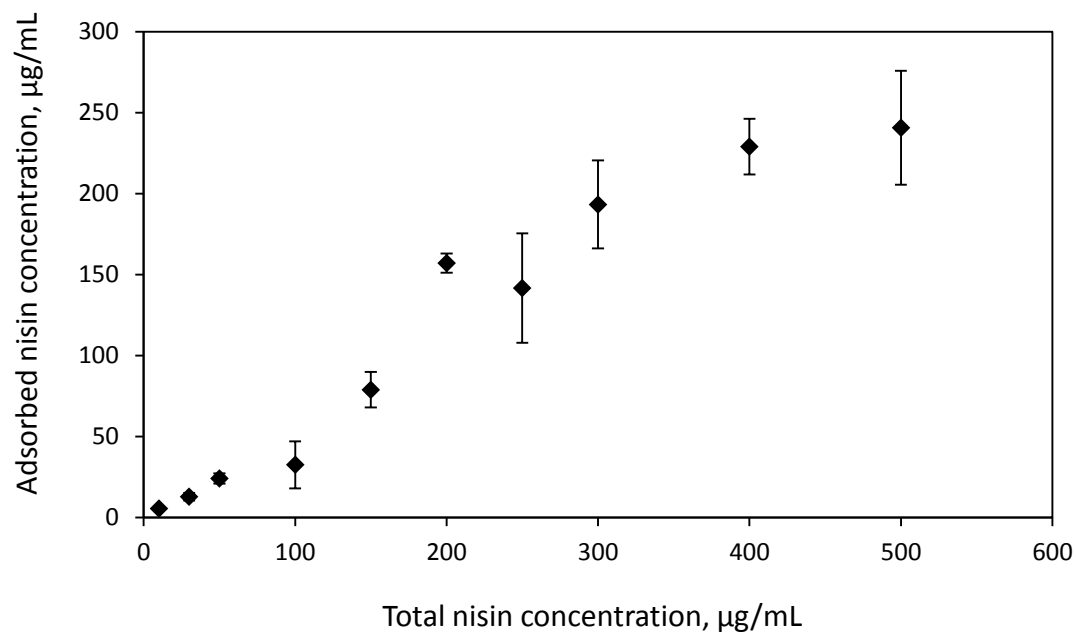


Figure 2.8 Nisin adsorption to PG-OS of DS 0.010 stabilized emulsion with nisin concentrations ranging from 10 to 500 µg/mL. Adsorbed nisin concentration (µg/mL) is plotted versus total nisin concentration (µg/mL). Mean values with error bars as standard deviation are shown (n=3).

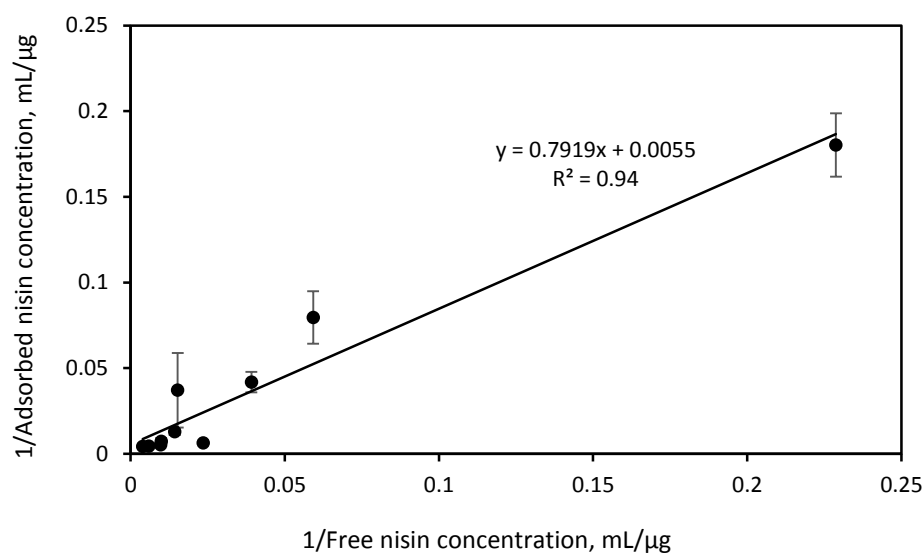


Figure 2.9 Linearized plots of (1/Average adsorbed nisin concentration) versus (1/Average free nisin concentration) to obtain monolayer adsorption capacity ( $Q_m$ ) and equilibrium constant value ( $K$ ) for PG-OS of DS 0.010 emulsion-nisin sample. Nisin concentrations ranges from 10 to 500  $\mu\text{g/mL}$ . Mean values with error bars as standard deviation are shown ( $n=3$ ).



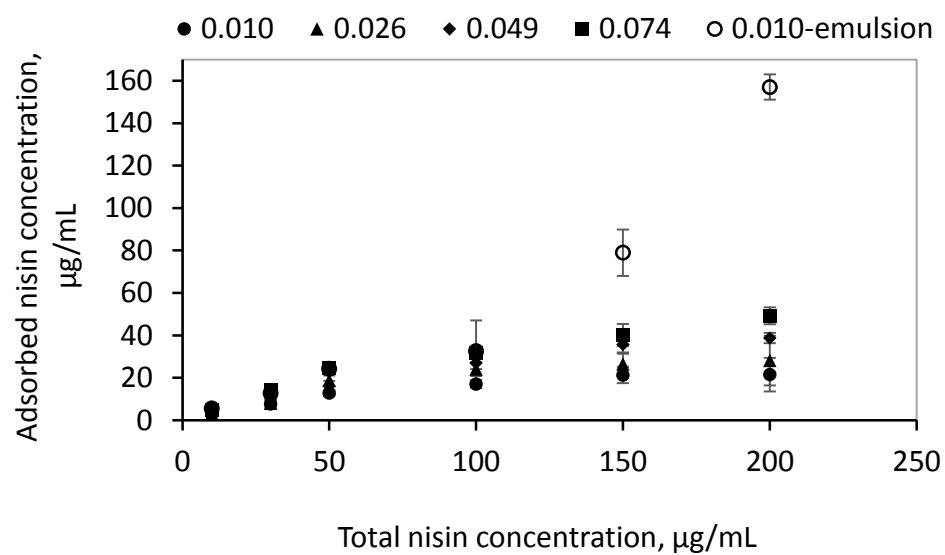


Figure 2.10 Adsorption plot of nisin to each of the four PG-OS nanoparticles with different degree of substitution (DS) in non-emulsion (aqueous) system (filled symbols) and PG-OS of DS 0.010 in emulsion phase (open symbols) with nisin concentrations ranging from 10 to 200 µg/mL. Mean values with error bars as standard deviation are shown (n=3).

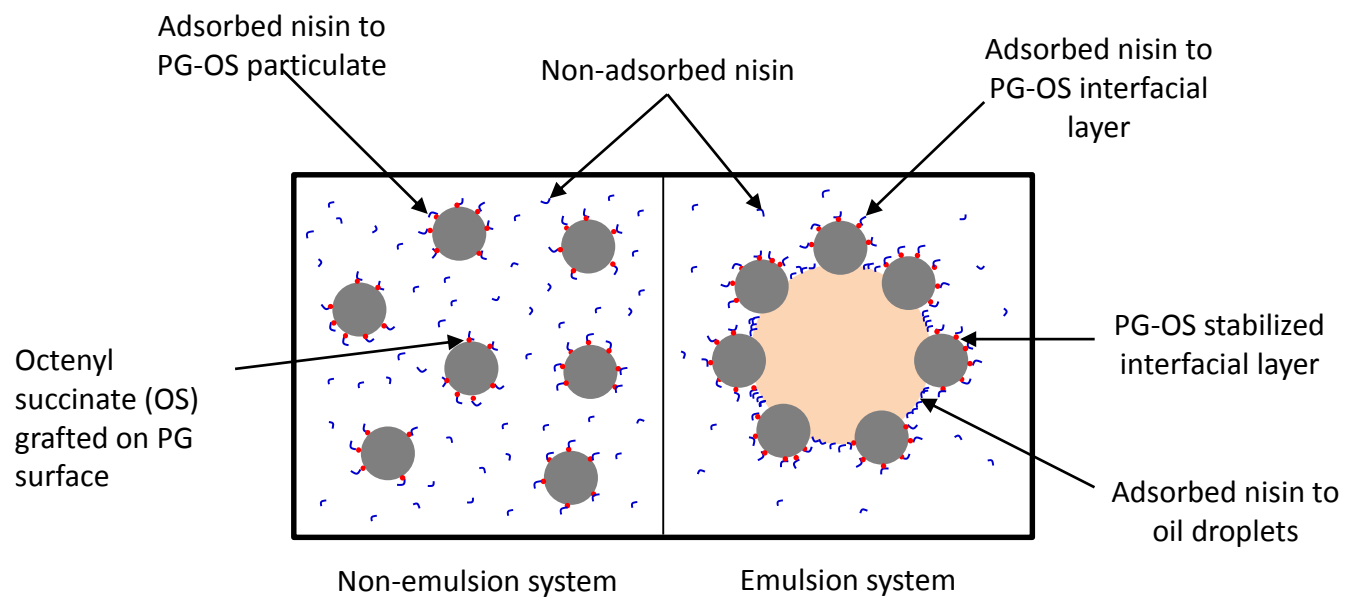


Figure 2.11 Schematic comparison between nisin adsorption to non-emulsion (aqueous) and emulsion system.

### CHAPTER 3. STARCH OCTENYL SUCCINATE STABILIZED EMULSION FOR THE PROTECTION OF NISIN ACTIVITY AGAINST *LISTERIA MONOCYTOGENES* IN A CANTALOUPE JUICE MODEL

#### 3.1 Abstract

Novel approaches geared towards enhanced food safety and security is the need of the hour. Food safety related to fresh and fresh-cut produce has remained a challenge due to increasing pathogen contamination and human deaths. Whole and fresh-cut produce including cantaloupes have been associated with a number of recent outbreaks. Nisin, an antibacterial peptide, has been used in the food industry for inhibiting Gram-positive pathogen such as *Listeria monocytogenes*. One strategy of protecting nisin activity over prolonged period of time is by its combination with a delivery agent such as oil-in-water emulsions. In this study, the protective effect of starch octenyl succinate (starch-OS) stabilized emulsion on nisin activity was evaluated in a food model (cantaloupe juice). Cantaloupe juice was selected because it can be used to simulate the hazardous environment of fresh-cut cantaloupe surface. The study demonstrated that protease enzyme activity in cantaloupe juice is the key factor responsible for depletion of nisin. Our research substantiated that nisin activity can be prolonged up to 6 days in a cantaloupe juice model against *Listeria monocytogenes* when combined with starch-OS based emulsion system. In addition, our study revealed that strong adsorption between positively charged nisin and negatively charged, starch-OS based colloidal assembly was the primary driving force behind the prolonged nisin activity.

**Keywords:** Starch octenyl succinate, emulsion, nisin, prolonged antimicrobial efficacy, *Listeria monocytogenes*

### 3.2 Introduction

Fresh and fresh-cut produce is an important area in food processing due to their high nutritional value. These convenient fresh foods have desirable nutritional profiles including antioxidant and free radical scavenging properties which make them highly desirable for consumption in home and food service operations. However, a number of food borne illnesses have been associated with such fresh and fresh-cut food systems. One of the greatest causes of food borne illnesses has been the fresh produce (Goodburn & Wallace, 2013). These include *E. coli* O157: H7 on strawberries in northwest Oregon resulting in 15 illnesses and 1 death and *Listeria monocytogenes* outbreak in chopped lettuce in California which resulted in a major recall (CDC, 2011). In terms of pathogen contamination of fresh produce, cantaloupes have probably suffered the most (Goodburn & Wallace, 2013). The greatest outbreak in cantaloupe industry has been the outbreak associated with *Listeria monocytogenes* in 26 states across US resulting in 130 illnesses and 30 deaths. In addition, another *Salmonella* outbreak originated in Southwest Indiana leading to 261 affected with 94 hospitalized and 3 deaths (CDC, 2011). The above statistics clearly indicates that novel preservation strategies need to be developed for improving cantaloupe safety in the US (CDC, 2012).

Cantaloupe belongs to the family of *Cucurbitaceae* and is one of the most popular fruits among American consumers (Ismail, Chan, Mariod, & Ismail, 2010). Cantaloupe fruit provides strong anti-oxidant and anti-inflammatory properties as it contains significant amounts of vitamin C, vitamin A and  $\beta$ -carotene, potassium and vitamin B6 (Solval, Sundararajan, Alfaro, & Sathivel, 2012; Vouldoukis, et al., 2004). On a daily value (DV) basis, one hundred gram of cantaloupe can provide 61.7% of vitamin C (36.7 mg) and 67.64% of vitamin A (3382 IU) (Solval, Sundararajan, Alfaro, & Sathivel, 2012). But cantaloupes (either whole or fresh-cut) have always been associated with contamination with both pathogenic and spoilage organisms. Fresh-cut cantaloupes possess a greater risk of pathogen contamination (leading to shorter shelf-life) and public health concerns. Fresh-cut cantaloupes, like many other fruits contain significant amounts

of fruit extract or juice which is rich in nutrients and enzymes. In addition, the pH of cantaloupe juice ranges between 5.6 to 6.3, water activity between 0.95 and 0.98, and brix between 8.50 and 11.40 making it ideal candidate for bacterial growth (Galeb, Wrolstad, & McDaniel, 2002; Sharma, Adler, Harrison, & Beuchat, 2005; Wang, et al., 2006). Currently, a lot of thermal and non-thermal processing methods (such as modified atmospheric packaging, UV light, irradiation, ozone processing and edible films and coatings) have been employed for shelf-life extension of cantaloupe and melon fruits and their juices, but their usage has resulted in the loss of essential nutrients (vitamins, color and degradation of aromatic compounds) and decrease in organoleptic properties (Martíñon, Moreira, Castell-Perez, & Gomes, 2014). Therefore, alternative strategies should be explored to enhance fresh-cut cantaloupe safety by the use of natural antimicrobial compounds such as peptides (for example, nisin).

Nisin is a positively charged lantibiotic peptide which is produced from *Lactococcus lactis* fermentation. It is made up of 34 amino acid moieties with an average molecular weight of 3.4 kDa and shows cationic and hydrophobic characteristics. It is able to bind to negatively charged cell membranes by nonspecific electrostatic interactions. Nisin kills Gram positive bacterial cells by a pore formation mechanism using a specific Lipid 2 docking molecule (Breukink & de Kruijff, 2006). Nisin being an antimicrobial peptide, can be easily depleted on cut cantaloupe surface due to presence of significant amount of proteolytic enzymes in juice. In order to protect nisin activity on cut cantaloupe surface for prolonged antimicrobial efficacy, it needs to be combined with a delivery vehicle such as oil-in-water emulsion system.

Previous research in our laboratory has shown that nisin can be protected and released for a prolonged period of time in a BHI-agar deep-well model by combining with carbohydrate nanoparticles such as phytoglycogen octenyl succinate (PG-OS) in both dispersion and emulsion based systems (Bi, Yang, Bhunia, & Yao, 2011a; Bi, Yang, Narsimhan, Bhunia, & Yao, 2011). BHI or brain heart infusion is a highly nutritious culture medium that supports the growth of both fastidious and non-fastidious organisms. Chemically, BHI comprises of calf brain, beef heart, peptones and proteose, dextrose, sodium chloride, and disodium phosphate and was used to mimic a real food system for

depletion of antimicrobials. It was confirmed that PG-OS-0.05 and PG-OS-0.12 materials when combined with 100 µg/mL of nisin showed antilisterial activity till 10 days in a BHI-agar deep-well model in aqueous dispersions. Similarly, PG-OS with DS of 0.013 when interacted with 150 µg/mL and 200 µg/mL nisin in the emulsion system showed antilisterial activity till 50 days in a BHI-agar deep-well model. In the current study, we use cantaloupe juice as the model to evaluate the prolonged protection and efficacy of nisin using a similar oil-in-water emulsion based delivery vehicle.

Carbohydrate or protein stabilized oil-in-water emulsion systems have been used extensively for the prolonged protection and delivery of active molecules. Starch is a polymer of D-glucose molecules. It consists of two types of structures: amylose (the linear component of starch joined by  $\alpha$ -1,4-D-glycosidic bonds) and amylopectin (the branched component of starch joined by both  $\alpha$ -1,4- and  $\alpha$ -1,6- glycosidic bonds). Chemical modifications are performed to such polysaccharides to impart useful functional properties. One common substitution to induce both negative charge and hydrophobicity to starch is octenyl succinylation. This modification results in the formation of starch octenyl succinate (Starch-OS). Amphiphilic properties in starch octenyl succinate allow it to be used in a wide range of applications including emulsification, microencapsulation, gel production and in films and coatings (Sweedman, Tizzotti, Schafer, & Gilbert, 2013). In this study, starch-OS was used over PG-OS because of its cost productive value and ease of availability.

The overall goal of this research was to study the prolonged protection of nisin using an oil-in-water emulsion system stabilized by starch-OS in a cantaloupe juice system against *Listeria monocytogenes*. The objectives of this study were to (a) understand the factor responsible for nisin depletion in cantaloupe juice, (b) study prolonged nisin protection against *Listeria monocytogenes* in the presence of depletion factors from cantaloupe juice and (c) understand the underlying mechanism for prolonged nisin activity when combined with an emulsion system.

### 3.3 Materials and Methods

#### 3.3.1 Materials

Starch octenyl succinate (starch-OS) was obtained from an industrial company. Tween-20 was purchased from Sigma Aldrich (St. Louis, MO). Soybean oil was purchased from local grocery store. Nisin was purchased from Sigma Aldrich (St. Louis, MO, 2.5% nisin and remaining 97.5% was milk solids). Brain heart infusion (BHI) and agar (BD Bacto) were purchased from BD Biosciences (Franklin Lakes, NJ). Dialysis membranes (MWCO: 300 kDa) were purchased from Harvard Apparatus (Holliston, MA).

#### 3.3.2 Starch-OS material characterization

Weight-average molar mass ( $M_w$ ) and root mean square radius ( $R_z$ ) of starch-OS were determined using high performance size exclusion chromatography connected with multi angle laser light scattering and refractive index detectors (HPSEC-MALLS-RI) (Wyatt Technology, Santa Barbara, CA). Two connected columns (PL Aquagel-OH 40 and 60) were used with a guard column (Polymer Laboratories, Varian Inc.). Molecular density ( $\rho$ ) was then calculated from  $M_w$  and  $R_z$  using the equation:  $\rho = \frac{M_w}{R_z^3}$ . The value of  $\rho$  is expressed in  $\text{g/mol}\cdot\text{nm}^3$  (Scheffler, Huang, Bi, & Yao, 2010).

To measure the degree of substitution (DS), the starch-OS material was dissolved in  $\text{D}_2\text{O}$  (Cambridge Isotope Laboratories, Andover, USA) at 5 mg/ml in order to exchange hydroxyl protons. The  $^1\text{H}$  NMR measurements were performed with a Bruker Avance DRX-500 NMR spectrometer operating at 499.89 MHz and equipped with a 5 mm inverse-detection triple-resonance Z-gradient probe. The DS of OS was calculated from integral values of the characteristic peaks of  $-\text{CH}_3$  ( $\sim 1.5\text{ppm}$ ) of OS and the sum of integral of anomeric proton of starch ( $\alpha$ -1,6 linkage, 5.6 ppm;  $\alpha$ -1,4 linkage 5.92 ppm) (Tizzotti, Sweedman, Tang, Schaefer, & Gilbert, 2011).

### 3.3.3 Starch-OS stabilized emulsions: preparation and characterization

Emulsion was prepared by dissolving 30.0 g starch-OS solid in 100.0 ml sodium acetate buffer (50 mM, pH 5.5). After complete dispersing, 30.0 g vegetable oil was added. The mixture was subjected to a high-speed homogenizer (Ultra Turrax, IKA T25 digital, NC, USA) at 18,000 rpm for 1 min, and a high-pressure homogenizer (GEA, PandaPlus, Italy) at 10,000 psi for 3 cycles to obtain a fine emulsion. Thereafter, the emulsion was heat treated using a boiling-water bath for 3 min before further testing.

To prepare nisin-containing emulsions, nisin stock (4.0 mg/mL) was diluted with emulsion to achieve concentrations of 100, 250, 500, 750 and 1,000 µg/mL. For each preparation, the concentration of starch-OS was maintained at 10.0 mg/mL. The zeta-potential values of mixtures were determined using a Zetasizer (ZS90, Malvern, Worcestershire, UK). The measurement was performed at 25 °C under an automatic setting condition with 1 min equilibration time. Zeta-potential is the electrostatic potential between the plane of shear (within the interfacial double layer) and the bulk fluid away from the interface. It is an important tool for understanding the interactions between charged groups and the stability of colloidal systems.

To determine the particle size distribution of oil droplets, the emulsion was diluted 100 times using sodium acetate buffer (50 mM, pH 5.5) and measured using the same Zetasizer.

### 3.3.4 Cantaloupe juice heating treatment and its impact on nisin activity

Cantaloupe juice was extracted from raw cantaloupes purchased from a local grocery store. The cantaloupe pulp was separated and crushed with a commercial blender (Waring Inc, USA), and centrifuged at  $10,000 \times g$  for 20 min. The supernatant was collected as cantaloupe juice, which was further sterilized using a 0.2 µm filter (Millipore, MA). This filter-sterilized aqueous content extracted from cantaloupe pulp is called cantaloupe juice hereafter. A portion of the juice was heat treated (20 min in boiling-water bath) to deactivate enzymes. To the native and heat-treated juices, emulsion with initial nisin preparation (500 µg/mL) was added in a ratio of 1:3 (nisin preparation: cantaloupe juice; v/v) and allowed to interact for 24 h at 4 °C under slow shaking



condition (60 rpm) (Lab-Line, Max/Rotator, USA). Aliquot of the mixtures were taken after 15 min, 12 h, and 24 h of interaction, and agar diffusion assay was used to determine the residual nisin activity using *Listeria monocytogenes* V7 (serovar 1/2a) as the target strain. The time periods for interaction were selected based on our preliminary tests.

For the agar diffusion assay procedure, 18.5 g of BHI medium was dispersed in 500.0 mL of distilled water. The pH of this dispersion was adjusted to 5.5 using concentrated HCl. To this mix, 3.75 g of agar and 5 g of Tween-20 was added and mixed. The dispersion was then autoclaved and poured on square Petri-plates (10 cm × 10 cm). BHI agar was also prepared in glass tubes using the similar protocol to form the top layer on Petri-plates as shown in Figure 3.1. Overnight grown *Listeria monocytogenes* ( $2 \times 10^8$  CFU/mL) culture was added into the tubes after cooling. The tubes were gently mixed and poured on the BHI agar plates to form a layer of bacteria (Fig. 1). After solidification for 10 min, 7.0 mm cork borer was used to form wells on the petri plates. 100  $\mu$ L of fluids were then transferred inside the wells. The antimicrobial loaded plates were incubated at 25 °C for 24 h and the diameter of inhibitory ring (D) was measured using a ruler. For measurement of ring size, three diameter readings (D) were taken and the actual size of inhibitory ring (x) was calculated by using the equation:  $x = (D-7)/2$  (Figure 3.1).

### 3.3.5 Cantaloupe juice: protease activity measurement

To determine the protease activity in cantaloupe juice, a protease fluorescent detection kit was used (Sigma Aldrich, St. Louis, MO). For the measurement, 20  $\mu$ L of incubation buffer, 20  $\mu$ L of FITC-casein substrate and 10  $\mu$ L solution (test, control or blank) were mixed together. The concentration of the control solution ranged from 0.3125 to 20.0  $\mu$ g/mL. Each tube was mixed gently and incubated for 4 hours at 37 °C in the dark. After incubation, 150  $\mu$ L of 0.6 N trichloroacetic acid (TCA) was added to each tube. The mixture was incubated again at 37 °C in the dark for 30 min, and then subjected to centrifugation at  $10,000 \times g$  for 10 min. After centrifugation, 10  $\mu$ L supernatant was withdrawn and mixed with 1.0 mL of the assay buffer. 200  $\mu$ L volume of the solution

(supernatant and assay buffer) was transferred in triplicates to black 96-well plates for reading the fluorescent intensity.

### 3.3.6 Adsorption of nisin in emulsion: equilibrium dialysis study

Equilibrium dialysis technique was employed to evaluate the interaction between starch-OS emulsion and nisin. The Fast Micro-Equilibrium Dialyzer unit (FMED) (Harvard Apparatus, MA) was used to extract and quantify nisin molecules in the non-adsorbed form. The FMED unit consists of 2 dialysis chambers separated by a membrane, each of volume 500  $\mu$ L. A cellulose acetate dialysis membrane with a molecular weight cut-off (MWCO) of 300 kDa was inserted in the dialysis unit with one closed chamber. With another chamber open, the unit was placed in the fluid in the vial and agitated for a certain period of time to reach equilibrium in a horizontal shaking water bath at 70 rpm. The total dialysis fluid was 100 mL and the volume of the dialysis chamber was 500  $\mu$ L; such a dialysis setting could be considered as nearly non-invasive (Figure 3.2).

Before performing the adsorption study, the time required for dialysis to reach its equilibrium was established. Equilibrium time was considered as the time needed for the internal dialysis chamber to reach the nisin concentration (500  $\mu$ g/mL) of the dialysis fluid. To determine the time needed to reach dialysis equilibrium, nisin (2.0 mg/mL) was interacted with heat treated cantaloupe juice (in a 1:3 v/v ratio, final nisin concentration was 500  $\mu$ g/mL) and transferred to glass vials with a total volume of 100.0 mL. Thereafter, equilibrium dialysis unit was placed inside the glass vials with its internal chamber loaded with sodium acetate buffer (50 mM, pH 5.5). Over several time intervals, aliquots were withdrawn from the internal chamber and the nisin concentration was determined using an agar diffusion assay with *Listeria monocytogenes* V7 as the target strain. Agar diffusion assay was performed over other chemical methods (e.g. Bradford Microassay procedure) for nisin quantification to eliminate the impact of other proteinaceous materials in the mixture, such as those from the cantaloupe juice.

To determine the concentration of non-adsorbed nisin in heat-treated juice that contained emulsion, equilibrium dialysis experiment was performed. Solutions were

prepared by mixing 25.0 mL (nisin alone solution or emulsion containing nisin) and 75.0 mL of heat-treated cantaloupe juice. Heat-treated juice was used for the equilibrium dialysis experiment for two reasons: (1) the antibacterial efficacy tests were performed with juice, and (2) there is no nisin depletion during dialysis process, which allowed for the determination of nisin amount.

### 3.3.7 Prolonged nisin activity against *Listeria monocytogenes*

To determine residual nisin activity, agar diffusion assay was employed. To prepare emulsion, 30.0 g starch-OS solid was dispersed in 100 mL sodium acetate buffer (50 mM, pH 5.5). To this dispersion, 30.0 g of vegetable oil was added. The mixture was subjected to high-speed homogenization at 18,000 rpm for 1 min (Ultra Turrax, IKA T25, NC, USA) and high-pressure homogenization at 10,000 psi for 3 cycles (GEA Panda Plus, Italy) to obtain the original emulsion. To 50.0 mL of this emulsion, 50.0 mL of nisin stock solution (4.0 mg/mL) was added and the mixture (2.0 mg/mL nisin) was placed at 4 °C for 24 hours for equilibration. To prepare nisin stock solution, 480.0 mg of nisin solid (Sigma Aldrich, containing 2.5% of pure nisin and 97.5% denatured milk solids) was dispersed in 3.00 mL of sodium acetate buffer (50 mM, pH 5.5). The dispersion was agitated (Lab-Line, Max/Rotator, USA) for 15 h at 70 rpm and 4 °C, and thereafter centrifuged at  $5000 \times g$  for 5 min at 15 °C. After centrifugation, the supernatant was collected as 4.0 mg/mL.

Starting from emulsion-based and nisin-alone preparations, two groups of working preparations were prepared: (1) Model group, in which both nisin preparations were further mixed with 3 volumes of fresh cantaloupe juice, and (2) Reference group, in which both nisin preparations were further mixed with sodium acetate buffer. All working preparations were stored at 4 °C. From each, 0.1 mL fluid was withdrawn after 15 min, 1, 2, 3 and 6 days. The aliquots were heat treated in a boiling water bath for 15 min before being subjected to BHI-agar diffusion bioassay in a procedure described earlier. The size of inhibition zone for each preparation was measured.

### 3.3.8 Statistical analysis

All data are expressed as mean  $\pm$  SD with sample size ( $n$ ) = 3. The scatter diagrams and bar charts were plotted using Microsoft Excel 2013 software. To evaluate the effect of formulation type, ANOVA analysis was performed ( $p < 0.05$ ) using SPSS software (version 21, IBM, USA). Tukey's HSD multiple comparison test was performed to evaluate the difference among different levels.

## 3.4 Results and Discussion

### 3.4.1 Characterization of starch-OS material

The  $M_w$ ,  $R_z$ , and  $\rho$  of starch-OS material were  $7.63 \times 10^5$  g/mole, 27.9 nm. and 38.3 g/mol·nm<sup>3</sup>, respectively. Starch-OS generally contains negligible quantities of amylose, the observed characteristics are mainly due to its amylopectin content. Amylopectin involves long chains which connects individual clusters (Scheffler, Huang, Bi, & Yao, 2010). These long chains provide flexibility to the starch-OS layer at the oil/water interface. Therefore, this flexible, chain-like conformation at the droplet surface with a negative charge density due to OS groups helps in adsorption of positively charged nisin. The average degree of substitution (DS) value of the starch-OS material was  $0.0217 \pm 0.002$  as showed by the NMR results.

### 3.4.2 Zeta-potential and particle size distribution of starch-OS emulsion

Figure 3.3 shows the effect of nisin addition to starch-OS stabilized emulsions. With 0, 100, 250, 500, 750, and 1000  $\mu\text{g/mL}$  of nisin incorporation, zeta-potential values of emulsions changed to -12.7, -6.7, -3.0, -1.2, -0.4, and +0.4 mV, respectively. Similar trend was observed in previous studies with the incorporation of nisin at 150 and 200  $\mu\text{g/mL}$  to waxy corn starch octenyl succinate (WCS-OS) emulsion, where the zeta-potentials were reported as -9 and -4 mV, respectively (Bi, Yang, Narsimhan, Bhunia, & Yao, 2011). Dynamic light scattering technique was employed to study the particle size distribution of oil droplets. The (Z-average  $D_H$  distribution) measured using dynamic light scattering technique was 261nm (Figure 3.4).

### 3.4.3 Protease in cantaloupe juice and its effect on nisin activity

Figure 3.5 shows the impact of heat treatment of juice on the depletion of nisin activity against *Listeria monocytogenes*. Like other fruit systems, cantaloupe and its juice contains significant amount of protease enzymes. This also includes a serine-protease enzyme, cucumisin (Murayama, Kato-Murayama, Hosaka, Sotokawauchi, Yokoyama, Arima, et al., 2012; Yamagata, Masuzawa, Nagaoka, Ohnishi, & Iwasaki, 1994). Both fresh and heat-treated cantaloupe juice was allowed to interact with nisin at 500 µg/mL level. The results showed that 15 min of incubation with juice did not affect the ring size. After 12 and 24 hours of interaction, the inhibitory ring reduced from 4.7 mm to undetectable size for non-heated juice and from 8.3 to 7.2 mm for heated juice, respectively. The juice alone treatment did not demonstrate any antilisterial activity, suggesting the complete absence of any antibacterial agent in cantaloupe juice. These tests suggested that the factor responsible for nisin depletion in juice can be completely removed by thermal treatment, and thus it should be positively correlated with its proteolytic activity.

Protease activity assay was performed using fluorescein isothiocyanate (FITC) casein as the substrate. The total protease concentration in fresh cantaloupe juice was 13.5 µg/mL, as shown by the protease activity assay. But, there was no detection of protease activity in the heat treated juice, suggesting that heat treatment was able to remove the proteolytic activity of cantaloupe juice. Therefore, the assay confirmed that cantaloupe juice proteases is responsible for nisin depletion.

### 3.4.4 Prolonged nisin activity in cantaloupe juice medium

Figures 3.6 and 3.7 depict the retention of nisin activity against *Listeria monocytogenes* V7. “Model” group refers to experiments performed in cantaloupe juice and “Reference” group refers to those in which nisin preparations were mixed with sodium acetate buffer (50 mM, pH 5.5). For the reference of nisin in aqueous solution, the size of inhibitory ring did not show any significant changes over the 6 days period, indicating high nisin stability during prolonged period of storage at refrigerated temperature. For the reference of nisin in emulsion preparation, there was an increase in

ring size during the first 24 hours followed by ring size reduction. This reduction in ring size was possibly due to a strong adsorption of nisin molecules at the interface of oil droplets. Similar reduction of nisin activity against *Listeria* was reported earlier for waxy corn starch-octenyl succinate (WCS-OS) stabilized emulsion (Bi, Yang, Bhunia, & Yao, 2011b). (WCS-OS) material has a high weight-average molar mass with  $M_w$  value of  $2.31 \times 10^7$  g/mole whereas the  $M_w$  of starch-OS preparation in the current study was  $7.63 \times 10^5$  g/mole. Due to larger molar mass, there is a greater abundance of carboxylate groups, that leads to extremely strong peptide adsorption at droplet surface. But the 100 times lower molar mass in case of the current starch-OS material was responsible for reduced abundance of adsorption sites, owing to a lesser degree of peptide adsorption. This difference in terms of peptide adsorption was also reflected by the zeta-potential values of starch-OS emulsion without nisin addition; -16.7 mV for WCS-OS and -12 mV for current starch-OS material. Therefore, the lower zeta-potential and weight-average molar mass value allowed for prolonged protection of nisin against *Listeria monocytogenes*.

The Model group demonstrated that starch-OS stabilized emulsion system could prolong nisin activity against *Listeria monocytogenes* in cantaloupe juice model. Figure 3.6 and 3.7 showed that in the cantaloupe juice, with a starting nisin concentration of 500 µg/mL, starch-OS stabilized emulsion was able to retain a detectable intensity of nisin activity till six days of interaction. After 6 days of incorporation with cantaloupe juice, the thickness of inhibitory ring for the starch-OS stabilized emulsion was 2.97 mm. For nisin aqueous solution in the Model group, there was rapid depletion of nisin activity, leading to a ring thickness of 3.60 mm after 1 days' incorporation. After 2 days of interaction with juice, the ring was non-detectable, suggesting negligible retention of nisin. ANOVA analysis revealed that all the treatments were significantly different ( $p < 0.05$ ). Tukey's HSD multiple comparison test showed that nisin in aqueous solution for model group was significantly different from the other three treatments ( $p < 0.05$ ).

### 3.4.5 Adsorption of nisin to starch-OS stabilized interface

Equilibrium dialysis was used to better understand nisin adsorption in starch-OS stabilized emulsion. To prepare dialysis fluids, 3 volumes of heat-treated juice were mixed with 1 volume of nisin-emulsion or nisin-alone preparations to achieve a nisin concentration of 500 µg/mL. The mixtures were subjected to dialysis for 40 h, and the nisin concentration of the permeate fluid, which was considered to be equal to the concentration of non-adsorbed nisin in the dialysis fluid, was quantified using BHI-agar diffusion bioassay against *Listeria monocytogenes*. The result showed that the ring size for non-adsorbed nisin was 1.75 mm and 6.67 mm for nisin-emulsion and nisin-alone preparations, respectively. Evidently, this result suggested a strong nisin adsorption in emulsion.

### 3.4.6 Mechanistic discussion

In a food system, the flow of nisin consumption is driven by the dynamics between peptide “depot” and “sink”, as well as the time in which such a flow may occur. In the model system of this study, the oil-water interface of starch-OS emulsion droplets served as depot of nisin molecules. The sink was associated with two events: (1) proteolytic degradation of nisin by enzymes in cantaloupe juice, which occurs all through that storage, and (2) complexation of nisin at the cell membrane of *Listeria*, which occurred only after the bacterial inoculation. In the presence of sink, there would be continuous consumption of total nisin from emulsion droplets and water phase.

In our designing of antimicrobial loaded delivery system, nisin was incorporated with the starch-OS stabilized emulsion, as shown in Figure 3.8. In Figure 3.8A, both nisin in aqueous solution and nisin in emulsion groups show negligible depletion due to absence of any depletion sinks. Figure 3.8B nisin aqueous solution group demonstrates rapid depletion of nisin activity due to proteolytic action of cantaloupe juice protease. Figure 3.8B emulsion combined nisin group demonstrates the protective effect of starch-OS interfacial layer on nisin activity. In the emulsion, immediately after nisin addition, certain fraction of the total added nisin binds with the interfacial layer due to electrostatic interaction and the residual nisin remains available in the aqueous system. Once, the nisin in emulsion mixture is added to the cantaloupe juice, protease enzyme demonstrates

proteolytic activity against the available nisin. There is depletion of the available nisin, but the remaining nisin activity is protected due to adsorptive binding to the emulsion interfacial layer. This degradation rate of the available nisin is proportional to, or at least positively correlated to the concentration of non-adsorbed nisin in the aqueous system. Figure 3.8C demonstrates the dynamics of the delivery system in the presence of bacterial cells. Bacterial cell membrane is made up of phospholipid bilayer and proteins that provide negative charge to the cell surface. This negatively charged cell membrane sets up a trigger mechanism that drives further nisin release from the oil/water interfacial layer. Therefore, in order to reach equilibrium, there is a continuous re-distribution of nisin molecules among the interfacial adsorption sites, emulsion aqueous phase and the bacterial cell membrane.

Different types of proteases are found in a variety of biological systems including fresh-cut fruits. Some of the major proteases found in fruits include the alkaline serine protease cucumisin in melons (honeydews and cantaloupes), thiol protease actinidin in kiwi fruit, and cysteine protease bromelain in pineapples (Katsaros, Katapodis, & Taoukis, 2009; Raimbault, Zuily-Fodil, Soler, Mora, & Cruz de Carvalho, 2013; Yamagata, Masuzawa, Nagaoka, Ohnishi, & Iwasaki, 1994). The most important physiological function of such proteases varies from peptic cleavage to comprehensive proteolysis. Therefore, this strategy of using starch-OS emulsion based delivery vehicle in protecting antimicrobial peptide against food borne pathogens can be applied to other fruit systems.

### 3.5 Conclusion

This study aimed at understanding the possibility of using starch octenyl succinate (starch-OS) stabilized oil-in-water emulsion for the protection of nisin activity against *Listeria monocytogenes* in a protease rich environment of cantaloupe juice. The study confirmed that the presence of protease enzyme in the cantaloupe extract was primarily responsible for rapid nisin depletion within 24 hours of interaction. However, the use of starch octenyl succinate stabilized oil-water emulsion system could be used to prolong



nisin activity after 6 days of interaction in a fresh cantaloupe juice system. In addition, equilibrium dialysis technique demonstrated that the adsorption between starch-OS colloidal assembly and nisin was the driving force behind nisin protection for a prolonged period of time.

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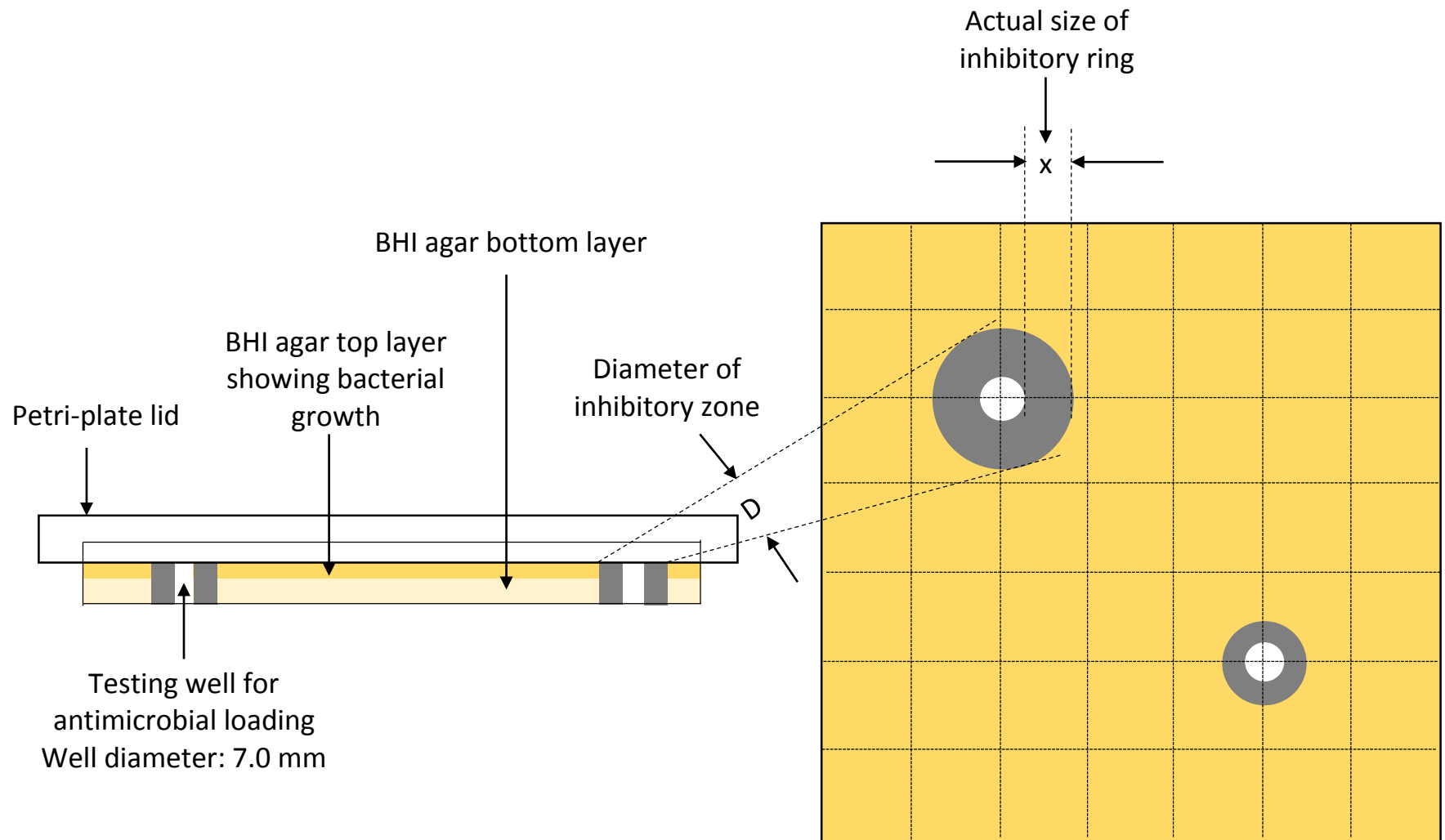


Figure 3.1 Schematic of agar diffusion bioassay for nisin quantification process. Left figure: Side view of a square petri-plate is shown. The top layer of BHI-agar shows the growth of *Listeria monocytogenes*. The bottom layer comprises of BHI agar. Right figure: Top view of a square petri-plate is shown with its grid lines. The transparent zone indicates the inhibitory effect of nisin on bacterial growth. D is the diameter of the inhibitory zone and x is the actual size of the inhibitory ring, where  $x = (D-7)/2$

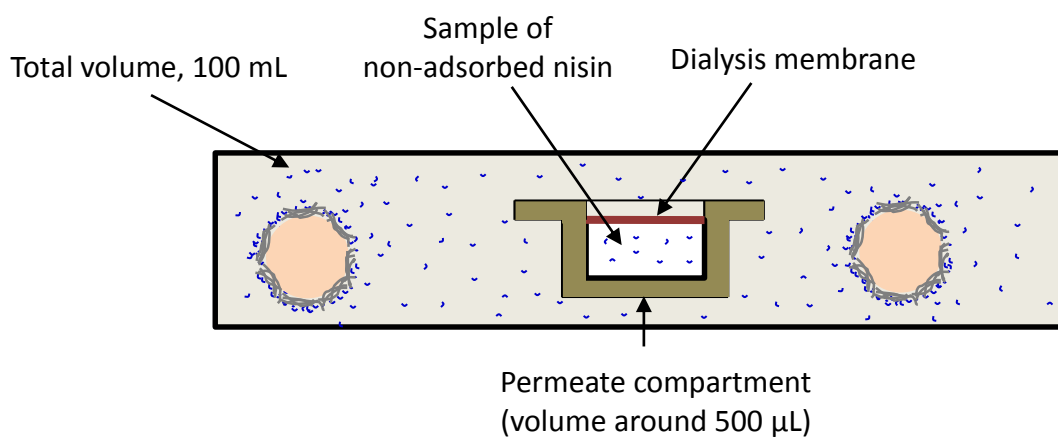


Figure 3.2 Fast Micro-Equilibrium Dialyzer (FMED) unit used for studying nisin adsorption phenomenon to starch-OS stabilized emulsion system.

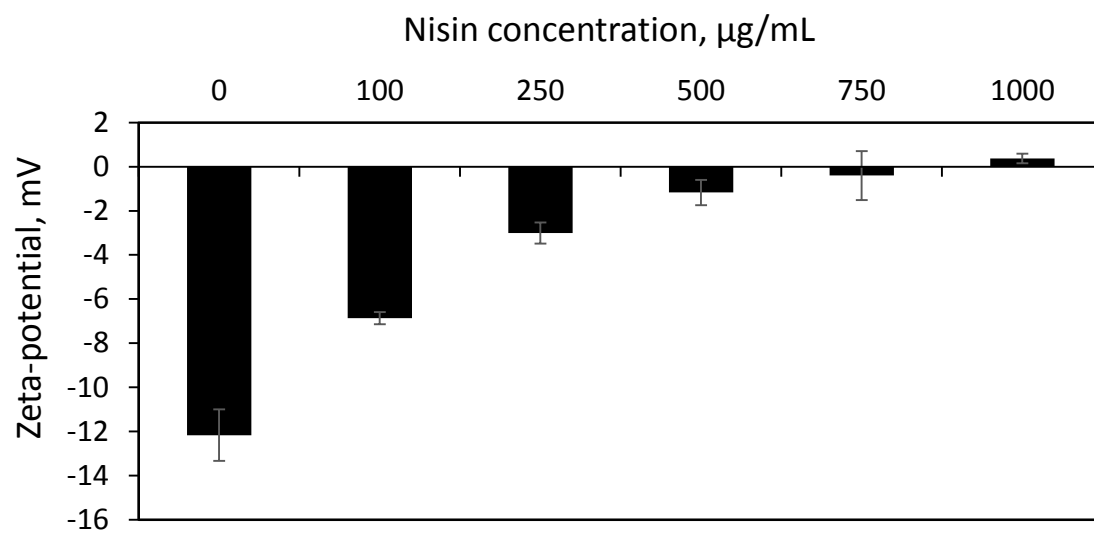


Figure 3.3 The impact of nisin addition on the zeta-potential of starch octenyl succinate (starch-OS) stabilized emulsion. Mean values with error bars as standard deviation are shown (n=3).

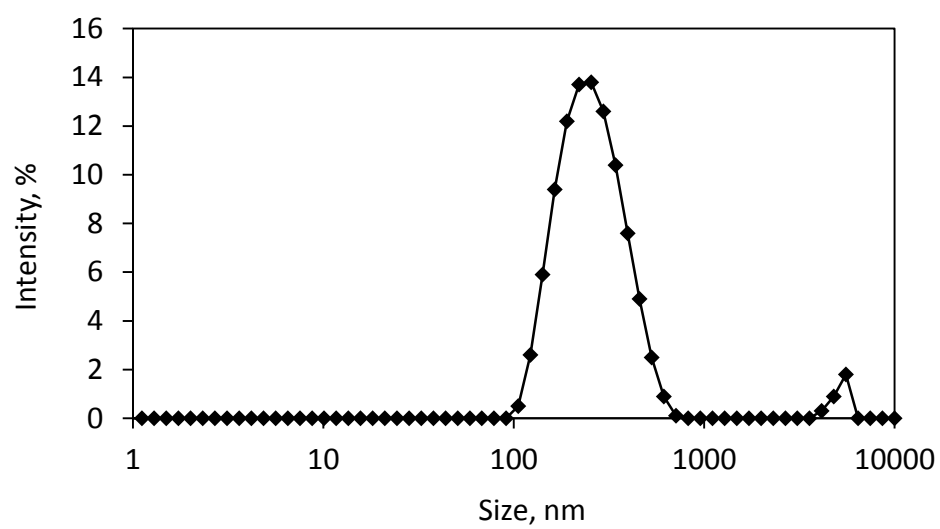


Figure 3.4 Particle size distribution (Z average  $D_H$ ) of starch-OS emulsion droplets



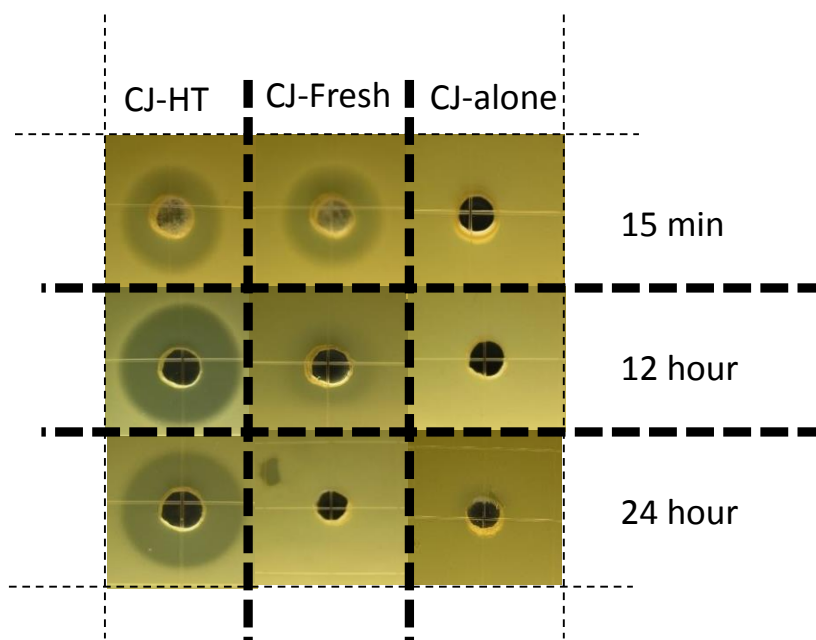


Figure 3.5 Inhibitory ring images to validate the effect of cantaloupe juice on nisin activity against *Listeria monocytogenes* V7. The 3 types of samples were CJ-HT: Cantaloupe juice-heat treated; CJ-Fresh: Cantaloupe juice-fresh and CJ-alone: Cantaloupe juice-alone. CJ-HT samples demonstrates strong antilisterial activity at 24 h, CJ-Fresh sample shows negligible zone of inhibition at 24 h. CJ-alone sample shows no antilisterial activity suggesting the absence of native antimicrobial components within cantaloupe juice.

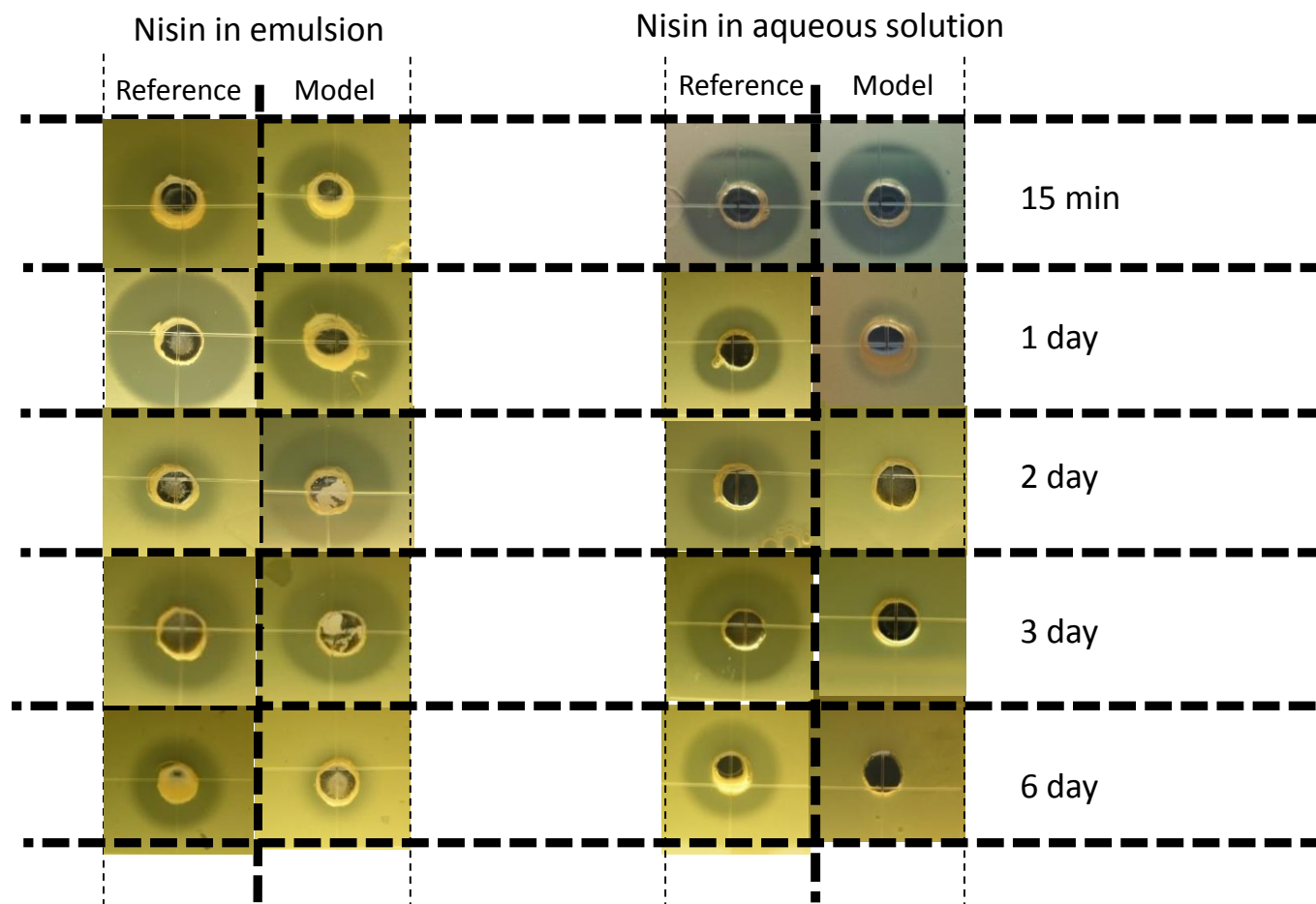


Figure 3.6 Images demonstrating protection of nisin activity against *Listeria monocytogenes* V7 by starch octenyl succinate (starch-OS) emulsion system at 4 °C storage. The starting nisin concentration was 500 µg/mL. The experiment was divided into two groups, (a) sodium acetate buffer and labeled as “Reference” and (b) fresh cantaloupe juice and labeled as “Model”. Nisin in emulsion refers to nisin incorporated with starch-OS emulsion and nisin in aqueous solution refers to nisin dissolved in sodium acetate buffer.

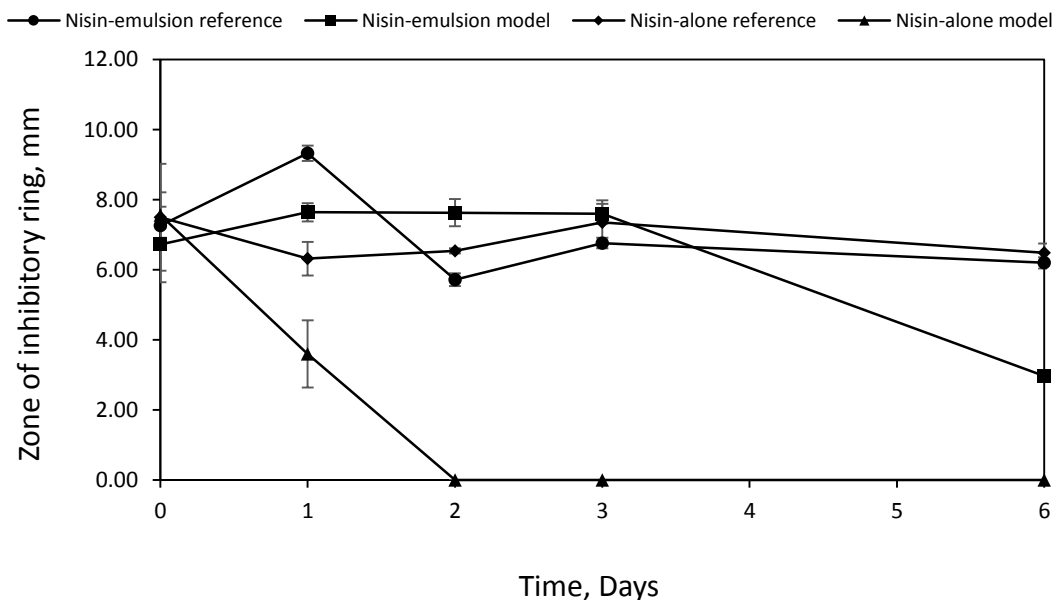


Figure 3.7 Preservation of nisin activity against *Listeria monocytogenes* V7 during 6 day 4 °C storage test in cantaloupe juice model. The time point 0 days on x-axis indicates 15 min of interaction with cantaloupe juice. Nisin in aqueous solution refers to nisin dissolved in sodium acetate buffer, nisin in emulsion refers to nisin incorporated with starch octenyl succinate (starch-OS) emulsion. Mean values with error bars as standard deviation are shown (n=3).

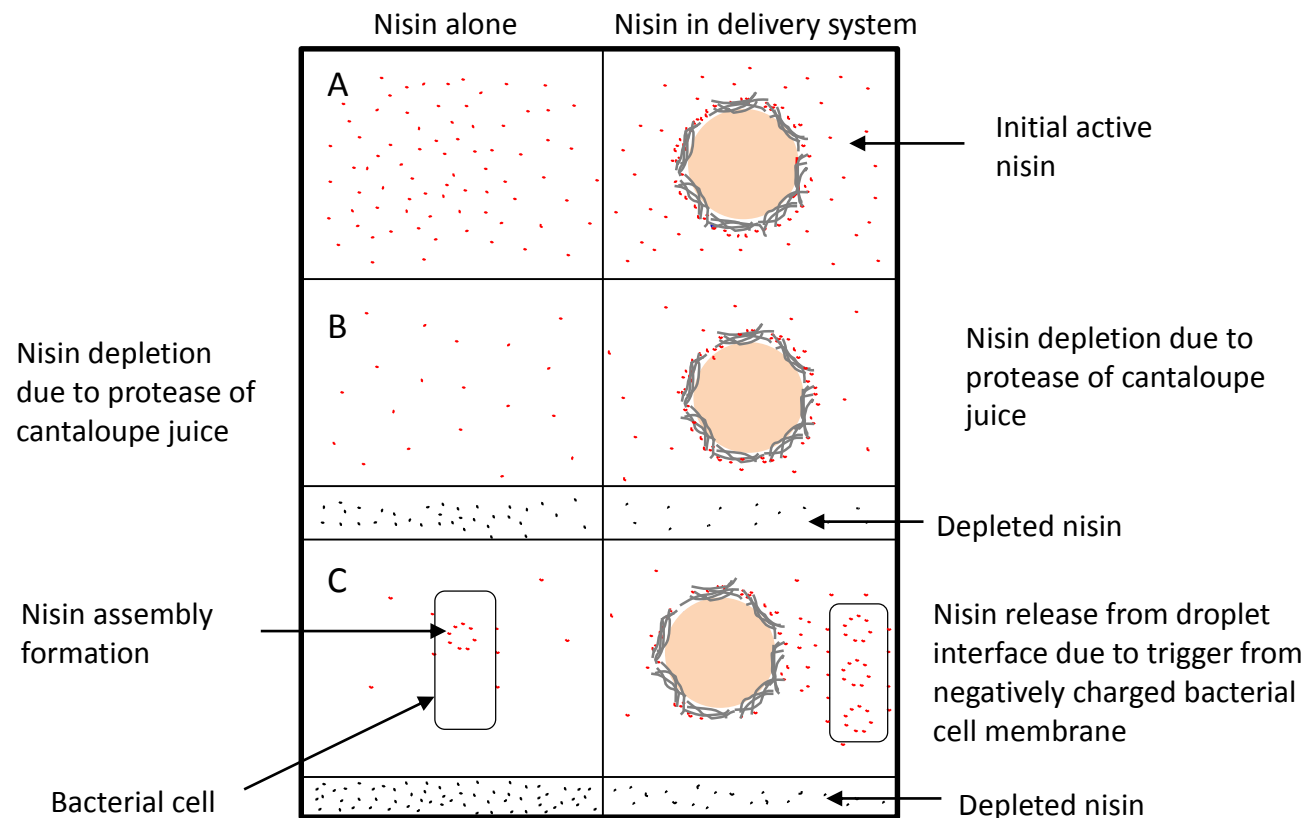


Figure 3.8 Schematic of nisin depletion, protection and release in non-emulsion (aqueous) and emulsion phases. In scenario (A), there is negligible depletion of nisin in both aqueous and emulsion groups due to absence of protease enzymes. In scenario (B) nisin alone solution is rapidly depleted due to the strong proteolytic activity of cantaloupe juice protease whereas interfacial layer in emulsion combined nisin protects nisin activity due to adsorption. In scenario (C) nisin alone solution group, there is absence of sufficient nisin concentration, thereby is incapable of inhibiting *Listeria* cells. In scenario (C) nisin in emulsion group, there is a continuous release of nisin molecules from the starch octenyl succinate (starch-OS) interfacial layer due to the presence of negatively charged cell membrane of *Listeria*, thereby providing sustained protection.

## CHAPTER 4. COMBINED ANTIMICROBIAL ACTIVITY OF NISIN AND THYMOL DELIVERED THROUGH A STARCH OCTENYL SUCCINATE STABILIZED EMULSION IN CANTALOUPE JUICE SYSTEM

### 4.1 Abstract

Natural antimicrobial compounds involved in food preservation are largely hindered by their rapid depletion by food components. In this study, oil-in-water emulsions formed with starch octenyl succinate (starch-OS) were used to stabilize nisin, an antimicrobial peptide and thymol, an antimicrobial essential oil. *Listeria monocytogenes* and *Salmonella* Typhimurium were used as model pathogens. Nisin and thymol were incorporated with emulsions and cantaloupe juice and tested for their antibacterial efficacies over 11 days of 4°C storage. The results showed that a combined use of nisin and thymol could slightly improve the antibacterial efficacy compared with individual uses. Nisin and thymol combination in non-emulsion and emulsion could generate 0.14 and 2.53 log<sub>10</sub> CFU/mL reduction against *Listeria*, respectively over 7 days of storage with cantaloupe juice. Similarly, emulsion formulations produced 2.80 log<sub>10</sub> CFU/mL reduction against *Salmonella* after 7 days, whereas non-emulsion efficacy was lost in first 2 days. More importantly, the use of emulsion was able to realize substantially enhanced antimicrobial efficacy. Combined formulations of nisin and thymol could generate 2.53 and 2.80 log<sub>10</sub> CFU/mL reduction against *Listeria* and *Salmonella* after 7 days of incubation, respectively. This study showed that starch-OS emulsion has the potential as a delivery system of antimicrobial components for prolonged pathogen inhibition.

**Keywords:** Starch octenyl succinate, oil-in-water emulsion, nisin, thymol.

## 4.2 Introduction

Outbreaks due to fresh and fresh-cut produce is increasing at an alarming rate in the United States. The major classes of bacteria that are responsible for many illnesses in fresh produce such as melons are *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* O157:H7 (Chen, Jin, Gurtler, Geveke, & Fan, 2012). U.S. Food and Drug Administration classifies *Listeria monocytogenes* as a “zero tolerance” organism in foods because it is responsible for fetal abortions, meningitis and septicemia in infants (Voetsch, Angulo, Jones, Moore, Nadon, McCarthy, et al., 2007). The mean annual cost due to *Listeria monocytogenes* infection was around \$2.04 billion. Similarly, fresh produce including cantaloupes have also been affected by *Salmonella* (Chen, Jin, Gurtler, Geveke, & Fan, 2012). Annual cost due to *Salmonella* (nontyphoidal) infection was around \$11.391 billion. In fact, one of the deadliest food borne pathogen attack was due to *Listeria monocytogenes* on cantaloupe melon in 2011 when 146 illnesses were reported causing 32 deaths and 1 miscarriage in 28 states (Upadhyay, Upadhyaya, Mooyottu, Kollanoor-Johny, & Venkitanarayanan, 2014).

Cantaloupe belongs to the family *Cucurbitaceae*, have been associated with contamination with pathogenic organisms (Fang, Liu, & Huang, 2013; Ismail, Chan, Mariod, & Ismail, 2010; Mahmoud, 2012). Currently, a lot of thermal and non-thermal processing technologies have been employed for shelf-life extension of cantaloupe and melon fruits, but their usage has resulted in the loss of essential nutrients and decrease in organoleptic properties (Martíñon, Moreira, Castell-Perez, & Gomes, 2014).

Nisin, a ribosomally synthesized antimicrobial peptide is produced by *Lactococcus lactis* bacterium. It is the only antimicrobial peptide (bacteriocin) which has been approved by the US FDA for use as a food preservative. Nisin kills Gram-positive bacteria very efficiently by a pore-forming mechanism, but is generally ineffective against Gram-negative bacteria. Therefore, in order to achieve nisin activity against Gram-negative bacteria, it needs to be combined with additional molecules. Thymol (2-isopropyl-5-methylphenol) is a major monoterpene phenol obtained from thyme (*Thymus*

*vulgaris*) essential oil and exhibits strong antibacterial effects against both Gram-positive and Gram-negative organisms (Burt, 2004). The antibacterial efficacy of thymol is primarily due to the presence of the hydroxyl group in the phenolic structure. In case of Gram-negative bacterial cell wall, thymol targets the outer membrane containing lipopolysaccharides (LPS) and thus enhances permeation of ATP. In the case of Gram-positive bacterial cell, thymol aligns itself within the fatty acids of the phospholipid membrane. This results in disintegration of the membrane structures and leads to increased cellular permeability (Burt, 2004; Lambert, Skandamis, Coote, & Nychas, 2001).

However, antimicrobial compounds suffer from rapid depletion within food systems. Nisin, for example may lose its antibacterial efficacy due to diffusion into the food mass or interactions with other food components such as enzymes (proteases), glutathione, sodium metabisulphite and titanium dioxide (Bhatti, Veeramachaneni, & Shelef, 2004; Jung, Bodyfelt, & Daeschel, 1992; Mahadeo & Tatini, 1994; Rose, Sporns, Stiles, & McMullen, 1999). On the other hand, thymol is a lipophilic compound that is highly volatile and is lost due to evaporation. In addition, thymol can also interact with hydrophobic food components such as proteins and lipids which can reduce its effective available concentration in the water phase (B. Shah, Davidson, & Zhong, 2013; B. Shah, P. M. Davidson, & Q. X. Zhong, 2012). Therefore, in order to protect antimicrobials against degradation factors in foods, it needs to be incorporated within a delivery agent such as an oil/water emulsion system.

Different types of delivery systems have been studied for the encapsulation of antimicrobials (including essential oil components and peptides). For example, essential oil components have been encapsulated within protein or polysaccharide matrices (Baranauskienė, Venskutonis, Dewettinck, & Verh  , 2006; Beristain, Garc  a, & Vernon-Carter, 2001) and emulsions (Suriyarak & Weiss, 2014). Specifically, thymol has been encapsulated in  $\beta$ -cyclodextrin (Ponce Cevallos, Buera, & Elizalde, 2010), zein based nanoparticles (Zhang, et al., 2014), whey protein isolate-maltodextrin conjugates (Bhavini Shah, Ikeda, Michael Davidson, & Zhong, 2012) and liposomes (Liolios, Gortzi, Lalas, Tsaknis, & Chinou, 2009). Nisin has also been combined with different

types of delivery systems such as films, nanoparticles, and liposomes (T. Jin, L. S. Liu, H. Zhang, & K. Hicks, 2009; Millette, Le Tien, Smoragiewicz, & Lacroix, 2007; Prombutara, Kulwatthanasal, Supaka, Sramala, & Chareonpornwattana, 2012; D. Xiao, Gommel, Davidson, & Zhong, 2011). Research in our lab demonstrated that nisin can be protected by combining with phytoglycogen octenyl succinate (PG-OS) nanoparticles in both non-emulsion and emulsion systems for extended period of time (Bi, Yang, Bhunia, & Yao, 2011a; Bi, Yang, Narsimhan, Bhunia, & Yao, 2011). Using a model depletion system, the PG-OS dispersion and emulsion systems retained nisin activity for up to 21 and 50 days in comparison with 7 and 15 days for nisin alone, respectively.

In addition to the use of single antimicrobial molecules, many studies have focused on their combined usage (S. Y. Lee & Jin, 2008; Solomakos, Govaris, Koidis, & Botsoglou, 2008a, 2008b; D. Xiao, Davidson, & Zhong, 2011; D. Xiao, Gommel, Davidson, & Zhong, 2011). Combination of antimicrobials can result in different types of response such as synergistic, partial synergistic, additive, indifference and antagonistic (Murdock, Cleveland, Matthews, & Chikindas, 2007). For example, thymol and nisin when combined together exhibited significant antibacterial efficacy against *Listeria monocytogenes* and *Escherichia coli* in TSB broth, minced beef and sheep meat (Govaris, Solomakos, Pexara, & Chatzopoulou, 2010; Solomakos, Govaris, Koidis, & Botsoglou, 2008a, 2008b). Similar studies have also been reported using combinations of nisin and  $\epsilon$ -polylysine against *Bacillus subtilis* (H. Liu, Pei, Han, Feng, & Li), mixture of essential oil fractions from lemon balm, marjoram, oregano and thyme against *Enterobacter* spp., *Listeria* spp., *Lactobacillus* spp., and *Pseudomonas* spp. (Gutierrez, Barry-Ryan, & Bourke, 2009), and combination of nisin and rhamnolipids against *Listeria monocytogenes* (Magalhães & Nitschke, 2013). Therefore, the overall goal of this study was to study the inhibition of *Listeria monocytogenes* and *Salmonella* Typhimurium using combined antimicrobial effects of nisin and thymol over a prolonged period of time delivered through an oil-in-water emulsion system in a cantaloupe juice model.



### 4.3 Materials and Methods

#### 4.3.1 Materials

Starch octenyl succinate (starch-OS) was obtained from an ingredient company. Tween-20 was purchased from Sigma Aldrich (St. Louis, MO). Soybean oil was purchased from local grocery store. Nisin was purchased from Sigma Aldrich (St. Louis, MO, containing 2.5% nisin and 97.5% milk solids). Thymol ( $\geq 99.5\%$ ) was purchased from Sigma Aldrich (St. Louis, MO). Brain heart infusion (BHI) and agar (BD Bacto) were purchased from BD Biosciences (Franklin Lakes, NJ).

#### 4.3.2 Characterization of starch-OS material

High performance size exclusion chromatography connected with multi angle laser light scattering and refractive index detectors (HPSEC-MALLS-RI) (Wyatt Technology, Santa Barbara, CA) was used to measure the weight-average molar mass ( $M_w$ ) and root mean square radius ( $R_z$ ) of starch-OS material. (PL Aquagel-OH 40 and 60) columns connected with each other were used with a guard column (Polymer Laboratories, Varian Inc.). Molecular density was then calculated from the molecular weight ( $M_w$ ) and root mean square-radius ( $R_z$ ) by using the following equation:  $\rho = \frac{M_w}{R_z^3}$ . The molecular density ( $\rho$ ) was expressed as  $\text{g/mol} \cdot \text{nm}^3$  (Scheffler, Huang, Bi, & Yao, 2010).

#### 4.3.3 Preparation of antimicrobial formulations

##### 4.3.3.1 Preparation of thymol and nisin aqueous solutions

Thymol stock solution was prepared by adding 2.0 g of thymol crystals to 1.0 L of sterile sodium acetate buffer (pH 5.5, 50 mM). The solution was gently stirred in dark for 24 h at 60 rpm on a hot plate (Cimarec Thermolyne, Barnstead International, IA) at 25 °C. After 24 h, the solution was centrifuged at  $7,800 \times g$  for 15 min twice to obtain a thymol concentration of 978.3  $\mu\text{g/mL}$  using HPLC analysis. Nisin stock solution was prepared by adding 1.2 g of nisin powder (2.5% nisin and 97.5% milk solids) in 3.00 mL

sterile sodium acetate buffer (pH 5.5, 50 mM). The dispersion was slowly agitated on a rotary shaker (Lab-Line, Max/Rotator, USA) for 15 h at 6 °C. Thereafter, the nisin dispersion was centrifuged at  $5000 \times g$  for 10 min to obtain nisin stock solution at 10.0 mg/mL (Bi, Yang, Narsimhan, Bhunia, & Yao, 2011). To prepare the solution that contained both nisin and thymol, 40  $\mu$ L of nisin stock solution (10.0 mg/mL) was added to thymol stock solution (978.3  $\mu$ g/mL) to obtain a final nisin concentration of 400  $\mu$ g/mL.

#### 4.3.3.2 Preparation of thymol and nisin emulsions

Starch-OS, soybean oil, thymol, and sodium acetate buffer (50 mM, pH5.5) were used to prepare thymol-containing oil-in-water emulsion. To prepare the aqueous portion, starch-OS (20.0 mg/mL) was dissolved in the buffer. To prepare the oil portion, thymol (20.0 mg/mL) was dissolved in soybean oil. Thereafter, the water portion and oil portion were mixed at a ratio of (1:1 v/v), the mixture was subjected to high-speed homogenization (Ultra Turrax, IKA T25 digital, NC, USA) at 18,000 rpm for 1 min, and high-pressure homogenization (GEA, PandaPlus, Italy) at 10,000 psi for 3 cycles to obtain a fine emulsion. Preliminary tests indicated that the homogenization resulted in a loss of around 50% of thymol owing to its highly volatile nature. Therefore, initially thymol concentration needed to be doubled to achieve approximately 1.0 mg/mL after homogenization. In the final emulsion, the total thymol concentration was 972.4  $\mu$ g/mL, with starch-OS (10.0 mg/mL) and oil (10.0 mg/mL).

To determine thymol concentration in the stock emulsion, 100  $\mu$ L of emulsion was added to 900  $\mu$ L of 100% ethanol, and the mixture was vortexed for 10 min and then centrifuged at  $12,000 \times g$  for 15 min to remove precipitated starch materials. The supernatant was used for thymol quantification as described later. The total thymol concentration in the emulsion was determined to be 972.4  $\mu$ g/mL using HPLC analysis. To prepare nisin in emulsion, a similar protocol was followed without thymol addition to soybean oil. To the emulsion formed, nisin stock solution (10.0 mg/mL) was added to achieve 400  $\mu$ g/mL, and the mixture was incubated for 24 h at 4 °C on a rotary shaker (Lab-Line, Max Rotator, USA). To incorporate both nisin and thymol in emulsion, nisin

stock solution was added to the thymol-containing emulsion to achieve 400 µg/mL and then the mixture was incubated for 24 h at 4 °C. The emulsions containing nisin, thymol and combination of nisin and thymol emulsions were immediately transferred to 50 mL tubes, capped and protected from light to prevent thymol evaporation and degradation. All formulated emulsion were heat treated using boiling water bath for 3 min before further tests.

The average particle size distribution of the different emulsion formulations in the presence of cantaloupe juice and heat-treated cantaloupe juice were measured using a Zetasizer (ZS90, Malvern, Worcestershire, UK) after 100 volumes dilution with sodium acetate buffer (50 mM/pH 5.5) for 11 days of incubation.

#### 4.3.4 Experimental design for antimicrobial tests

Cantaloupe juice was extracted from raw cantaloupes purchased from a local grocery store. The cantaloupe pulp was separated and crushed with a commercial blender (Waring Inc, USA). Then the pulp was centrifuged at  $7,800 \times g$  for 20 min (Beckman Coulter, CA). The supernatant was collected as cantaloupe juice, which was sterilized by using a 0.2 µm filter (Millipore, MA). Antimicrobial evaluations were performed in two groups: (1) model (in the presence of depletion factors) and (2) reference (in the absence depletion factors). In the model group, proteolytic enzymes from cantaloupe juice and chamber headspace for evaporation were the factors for the depletion of nisin and thymol, respectively. For the reference group, the proteolytic enzymes in the juice were denatured by heating (boiling-water bath, 20 min), and the preparations were placed in full volume in 50-mL tubes to eliminate the effect of headspace evaporation.

To determine the protease activity in cantaloupe juice, a protease fluorescent detection kit was used (Sigma Aldrich, St. Louis, MO). For the measurement, 20 µL of incubation buffer, 20 µL of FITC-casein substrate and 10 µL solution (test, control or blank) were mixed together. The concentration of the control solution ranged from 0.3125 to 20.0 µg/mL. Each tube was mixed gently and incubated for 4 hours at 37 °C in the dark. After incubation, 150 µL of 0.6 N trichloroacetic acid (TCA) was added to each

tube. The mixture was incubated again at 37 °C in the dark for 30 min, and then subjected to centrifugation at  $10,000 \times g$  for 10 min. After centrifugation, 10  $\mu$ L supernatant was withdrawn and mixed with 1.0 mL of the assay buffer. 200  $\mu$ L volume of the solution (supernatant and assay buffer) was transferred in triplicates to black 96-well plates for reading the fluorescent intensity.

Table 4.1 shows different formulations with their corresponding environment and nomenclatures. For the model and reference groups, the following formulations were prepared: nisin in aqueous solution (N-A-model, N-A-ref); thymol in aqueous solution (T-A-model, T-A-ref); combination of nisin and thymol in aqueous solution (NT-A-model, NT-A-ref); nisin in emulsion (N-E-model, N-E-ref); thymol in emulsion (T-E-model, T-E-ref); combination of nisin and thymol in emulsion (NT-E-model, NT-E-ref); and negative control comprising of sodium acetate buffer (Buffer-model, Buffer-ref). Model formulations were mixed with cantaloupe juice (1:1, v/v) and placed in 1-liter sterile glass bottles (Figure 4.1). Reference formulations were mixed with heat-treated cantaloupe juice (1:1, v/v) and placed in full in 50-mL tubes. All preparations were stored at 4 °C for 11 days.

Twelve-inch sterile needles (Figure 4.1) were used to withdraw fluids from the glass bottles. To maintain the sterile conditions in the bottles, the punctured caps were sealed with elastic polytetrafluoroethylene film. After 30 min and 1, 3, 5, 7, and 11 days of incubation, fluids were withdrawn from individual glass bottles and tubes, and each was divided in two portions. Portion-1 was subjected to immediate antibacterial evaluations. For portion-2, the fluid was subjected to 80 °C heating for 15 min to deactivate protease and placed in a -80 °C freezer for later quantification of nisin and thymol.

#### 4.3.5 Antimicrobial efficacy tests

After incubation in the respective model or reference groups, one milliliter of the fluids were transferred to reaction tubes. 10  $\mu$ L of inoculum from a  $1 \times 10^8$  CFU/mL overnight grown *Listeria monocytogenes* V7 culture were added in the tubes to obtain a starting bacterial load of  $1 \times 10^6$  CFU/mL. Similar procedure was followed for

*Salmonella* Typhimurium to obtain a starting bacterial load of  $1 \times 10^6$  CFU/mL. The reaction tubes containing antimicrobial preparations and inoculum was stored at 4 °C for 20 h on a rotary shaker at 60 rpm (Lab-Line, Max Rotator, USA). Low temperature of incubation was selected in order to simulate the growth of *Listeria* and *Salmonella* in foods stored under refrigerated conditions. 100 µL aliquot were withdrawn from each tube and diluted in PBS buffer. Then 100 µL of liquid was pour plated on BHI agar, incubated at 37 °C for overnight and enumerated. The colony counts were reported as mean  $\log_{10}$  CFU/mL from three replicates.

#### 4.3.6 Thymol quantification in aqueous and emulsion formulations

The aliquots collected after 30 min, 1, 3, 5, 7 and 11 days of storage were subjected to HPLC to determine thymol content. The HPLC system was equipped with a diode array detector, a quaternary pump, and a Supelcosil LC-18-DB column (3 µm, 15 × 4.6 mm). HPLC grade water mixed with acetonitrile (50:50 v/v) was used as the mobile phase in an isocratic mode at a flow rate of 1.00 mL/min. The sample injection volume was 10 µL. UV spectra was recorded at a wavelength of 274 nm. The data were analyzed using Chemstation software Rev. B.01.03 (Agilent Technologies, CA). A calibration curve was prepared using pure thymol standard with concentrations ranging between 10 and 500 µg/mL in a solvent that was prepared by mixing 90% ethanol and 10% sodium acetate buffer (50 mM, pH 5.5) in volume. For thymol quantification, each aliquot withdrawn during the 11-day storage were dispersed in a solvent that contained 90% ethanol and 10% sodium acetate buffer (50 mM, pH 5.5).

To determine the total thymol concentration in various preparations, 100 µL of each heat-treated fluids (solutions or emulsions) were mixed with 900 µL of 100% ethanol. The mixture was vortexed for 30 min and centrifuged at  $12,000 \times g$  for 15 min to remove precipitates (mostly starch-OS). The supernatant was subjected to HPLC analysis.

To determine thymol amount in the aqueous phase of emulsion, the fluids were subjected to ultracentrifugation ( $75,000 \times g$ , 15 min) and the serum was collected for thymol quantification. The ultracentrifugation speed and time was established through a

set of preliminary experiments. The emulsions were subjected to centrifugation at various relative centrifugal forces (10,000 to  $75,000 \times g$ ). Collected serums were observed under an upright optical microscope; at  $75,000 \times g$  (15 min) where negligible oil droplets were observed. For each supernatant collected from thymol-containing emulsions, 100  $\mu\text{L}$  was withdrawn, mixed with 900  $\mu\text{L}$  ethanol, and subjected to centrifugation to remove precipitate (mostly starch-OS). The supernatant was subjected to HPLC analysis to determine thymol amount.

#### 4.3.7 Water phase nisin quantification

For preparations that did not contain thymol, nisin quantification was conducted using the agar diffusion bioassay with *Listeria monocytogenes* V7 as the testing strain. For those that contained thymol, the fluids were firstly subjected to nitrogen gas flushing to completely remove thymol before conducting nisin diffusion bioassay. Thymol removal was needed to make sure that the inhibitory ring in the diffusion assay was attributed only to nisin.

Nitrogen gas flushing was conducted using a Reacti-Vap iii module (Thermo Fisher Scientific, PA) using nitrogen gas (1 psi) through stainless steel needles. Based on preliminary tests, a flushing time over 30 min was sufficient to reduce the thymol concentration below 10  $\mu\text{g/mL}$  when the total volume used for flushing was 500  $\mu\text{L}$  and a starting thymol concentration was 615  $\mu\text{g/mL}$ . In actual treatment, the flushing was performed for 50 min. Thereafter, each flushed solution was mixed with sodium acetate buffer (50 mM, pH 5.5) to make up its original weight. From all solutions (T-A-model, NT-A-model, T-E-model, NT-E-model, T-A-ref, NT-A-ref, T-E-ref and NT-E-ref), aliquots were withdrawn for evaluating residual thymol using HPLC analysis. It was shown that the nitrogen gas flushing was able to reduce thymol concentration to less 5  $\mu\text{g/mL}$  for even the fluid that contained potentially the highest amount of thymol (Figure 4.4). The flushed fluids were then subjected to nisin bioassay along with those that did not contain thymol in formulations.

For the agar diffusion assay procedure, 18.5 g of BHI medium was dispersed in 500.0 mL of distilled water. The pH of this dispersion was adjusted to 5.5 using

concentrated HCl. To this mix, 3.75 g of agar and 5.0 g of Tween-20 was added and mixed. The dispersion was then autoclaved and poured on square Petri-plates (10 cm × 10 cm). BHI agar was also prepared in glass tubes using the similar protocol to form the top layer on Petri-plates as shown in Figure 4.2. Overnight grown *Listeria monocytogenes* ( $10^8$  CFU/mL) culture was added into the tubes after cooling. The tubes were gently mixed and poured on the BHI agar plates to form a layer of bacteria (Figure 4.2). After solidification for 10 min, 7.0 mm cork borer was used to form wells on the petri plates. 100.0  $\mu$ L of fluids were then transferred inside the wells. The antimicrobial loaded plates were incubated at 25 °C for 24 h and the diameter of inhibitory zone (D) was measured using a ruler. For quantification of actual ring size, three diameter readings (D) were taken and the size of inhibitory ring (x) was calculated by using the equation:  $x = (D-7)/2$ . From three readings of x, the average value was calculated.

#### 4.3.8 Statistical analysis

All data are expressed as mean  $\pm$  SD with sample size (n) = 3. A probability value (p-value) of 0.05 was used for statistical significance for all the experiments. ANOVA analysis was performed using SPSS software (version 21, IBM). Tukey's multiple HSD comparison test was performed to evaluate significant differences among treatments.

### 4.4 Results

#### 4.4.1 Physical and structural characterization of starch-OS material

The weight-average molar mass ( $M_w$ ) and root mean square radius ( $R_z$ ) of the starch-OS materials were  $7.63 \times 10^5 \pm 0.03511$  g/mole and  $27.9 \pm 4.11$  nm, respectively. The average molecular density ( $\rho$ ) of starch-OS was calculated as  $38.3 \pm 16.39$  g/mol·nm<sup>3</sup> similar to that of dispersed amylopectin (Huang & Yao, 2011). The degree of polymerization (DP) of the starch-OS material was calculated as  $4711.9 \pm 21.7$ .

#### 4.4.2 Particle size and stability of emulsions

Figure 4.3 depicts the average particle size of the various emulsion formulations (model and reference) over the 11 day storage period. In the model group, the average particle size for N-E-model (nisin in emulsion-model), T-E-model (thymol in emulsion-model) and NT-E-model (combination of nisin and thymol in emulsion-model) ranged between 510 to 482 nm, 491 to 501 nm and 478 to 498 nm, respectively. For the reference group, the average particle size for N-E-ref (nisin in emulsion-reference), T-E-ref (thymol in emulsion-reference) and NT-E-ref (combination of nisin and thymol in emulsion-reference) formulations ranged between 525 to 625 nm, 528 to 606 nm and 547 to 607 nm, respectively. The minor variation in particle size distribution over the 11 day storage period shows the high stability of starch-OS stabilized emulsions in the presence of fresh and heat-treated cantaloupe juice.

#### 4.4.3 Retention of nisin in non-emulsion and emulsion systems

##### 4.4.3.1 Non-emulsion: Model and reference

The reduction in residual thymol concentrations after nitrogen flushing for combined formulations (NT-A-ref, NT-A-model, NT-E-ref and NT-E-model) is shown in Figure 4.4. In the reference group, nisin non-emulsion formulations (N-A-ref and NT-A-ref) in the presence and absence of thymol, demonstrated minor depletion when interacted with heat treated cantaloupe juice (Figure 4.5a). The zone of inhibitory ring in N-A-ref maintained from 5.87 mm (30 min) to 5.87 mm (7 days) of incubation. Similarly, the nisin concentration in NT-A-ref decreased from 6.11 mm (30 min) to 5.87 mm (7 days). This minor reduction in ring size demonstrates the high stability of nisin under refrigerated conditions of storage in the absence of depletion factors. In the model group, both N-A-model and NT-A-model demonstrated extensive depletion of nisin concentration in the first 2 days of storage with cantaloupe juice. In N-A-model, zone of inhibitory ring depleted from 6.34 mm (30 min) to negligible concentration (7 day). Similarly, for NT-A-model, the zone of inhibitory ring decreased from 6.97 mm (30 min) to negligible concentration (7 day) (Figure 4.5a).



#### 4.4.3.2 Emulsion: Model and reference

For the emulsion formulations (N-E-ref, NT-E-ref, N-E-model and NT-E-model), zone of inhibitory ring in the emulsion water phase was quantified after ultracentrifugation process. In the reference group (N-E-ref and NT-E-ref) the zone of inhibitory ring was maintained from 4.40 (30 min) to 4.35 mm (7 days), respectively (Figure 4.5b). In the model group, zone of inhibitory ring in N-E-model showed a declining trend from 4.32 mm (30 min) to 2.76 mm (7 days) of storage with cantaloupe juice. Similarly, the nisin concentration in NT-E-model formulation was reduced from 4.72 mm (30 min) to 4.08 mm (7 days) after incorporation with cantaloupe juice (Figure 4.5b).

#### 4.4.4 Retention of thymol in non-emulsion and emulsion systems

##### 4.4.4.1 Non-emulsions: Model and reference

The following definitions are used in the study regarding thymol quantification: (a) Non-emulsion (aqueous) formulations: Total thymol concentration (TTC) in non-emulsion (aqueous) formulations refers to the thymol present in the liquid at a given time point. Thymol concentration in aqueous formulation T-A-model, NT-A-model, T-A-ref and NT-A-ref refers to total thymol. (b) Emulsion formulations: Total thymol concentration (TTC) in emulsion formulations refers to the sum of thymol in oil droplets and the emulsion water phase. Thymol concentration of emulsion serum (STC) refers to the concentration in water obtained after the ultracentrifugation process. For thymol concentration in emulsion formulations T-E-model, NT-E-model, T-E-ref and NT-E-ref, total thymol concentration (TTC) and serum thymol concentration (STC) were quantified.

For thymol in aqueous solution (formulation T-A-ref in Table 1), the residual concentration decreased from an initial value of 493 µg/mL (30 min evaporation) to 461 µg/mL (5 days) and 438 µg/mL (11 days) (Figure 4.6a). Similarly for formulation NT-A-ref, there was no significant change of thymol concentration during the incubation period. On the other hand, the model tests were performed in a 1000 mL glass chamber with a

950 mL headspace volume, which was used for thymol evaporation. For formulation T-A-model, there was rapid depletion of TTC from 446 to 3 µg/mL after 5 days evaporation within the experimental chamber ( $p < 0.05$ ). Similar trend was also observed in formulation NT-A-model ( $p < 0.05$ ) (Figure 4.6a).

#### 4.4.4.2 Emulsion: Model and reference

The distribution of antimicrobials in the emulsion is a governing factor for antimicrobial efficacy. This distribution of thymol in the emulsion oil and water phases is driven by its partition coefficient ( $P_{o/w}$ ). In general,  $P_{o/w}$  can be defined as the ratio between the equilibrium concentrations of a solute in two immiscible liquids, in the limit of zero concentration (Han & Washington, 2005; Viljanen, Kylli, Hubbermann, Schwarz, & Heinonen, 2005). Mathematically, oil-water partition coefficient ( $P_{o/w}$ ) is:  $P_{o/w} = \frac{C_{oil}}{C_{water}}$ , where  $C_{oil}$  is the molar concentration of the solute in oil phase at a specific temperature and  $C_{water}$  is the molar concentration of the solute in the water phase (Han & Washington, 2005). Therefore oil-water partition coefficient for thymol in the present study is mathematically defined as,  $P_{o/w} = \frac{Thymol_{oil}}{Thymol_{water}}$ .

For emulsion formulations T-E-ref and NT-E-ref, the total (TTC) and serum thymol (STC) concentrations were quantified to obtain  $P_{o/w}$  value. As expected, both the total and serum thymol concentrations did not show significant change over the 11 day period due to minimal thymol volatilization. The STC of formulations T-E-ref and NT-E-ref decreased from 167 to 125 µg/mL and 183 to 156 µg/mL, respectively (Figure 4.6b). From the TTC and STC, thymol concentration in the oil droplets was calculated by method of difference. Table 4.2 shows the partition coefficient ( $P_{o/w}$ ) values of the various formulations. For NT-E-ref formulation, the  $P_{o/w}$  increased from 1.394 (30 min evaporation) to 1.937 (5 days) and 2.006 (11 days) ( $p > 0.05$ ). The percent thymol released from the oil to emulsion water phase indicated a minor decrease from 35.5 to 33.5 % of TTC for T-E-ref formulation with the progress of depletion time from 30 min to 11 days. Similarly, NT-E-ref showed a decreasing trend in percent thymol from 42.2 to 34.8 % of TTC over the 11 day period.

For formulation T-E-model the emulsion could retain around 40 µg/mL thymol in the serum after 7 days of incubation ( $p < 0.05$ ) (Figure 4.6b). The  $P_{o/w}$  for T-E-model indicated an increasing trend from 1.448 to 2.143 and 2.724 through the 11 days of storage (Table 4.2) ( $p > 0.05$ ). Formulation NT-E-model demonstrated the highest retention of thymol in the water phase of emulsion (51 µg/mL). Thymol concentration in the oil phase was calculated by the method of difference. The  $P_{o/w}$  value decreased from 1.396 to 1.369 and 0.965 over 30 min, 5 and 11 days of depletion for NT-E-model (Table 2) ( $p > 0.05$ ). The percent thymol released from the oil to emulsion serum also showed a decreasing trend for T-E-model. Interestingly, for NT-E-model, the percent thymol released in serum increased from 43.8 to 67% of TTC.

#### 4.4.5 Combined uses of nisin and thymol and their antibacterial efficacy against *Listeria monocytogenes* and *Salmonella* Typhimurium

##### 4.4.5.1 *Listeria monocytogenes*: Non-emulsion systems

The non-emulsion (aqueous) formulations in the reference group demonstrated strong antibacterial efficacy against *Listeria monocytogenes* V7 during the 11 day storage period (Figure 4.7 a and b). The *Listeria* count in the negative control was maintained between 6 to 6.32 log<sub>10</sub> CFU/mL. All the formulations in the reference group demonstrated a minimum of 3.0 log<sub>10</sub> CFU/mL difference. N-A-ref and T-A-ref recovered from 1 to 3.26 log<sub>10</sub> CFU/mL and 1.9 to 3.10 log<sub>10</sub> CFU/mL over the 11 day period of incubation with heat treated cantaloupe juice (Figure 4.7a). *Listeria* count in NT-A-ref formulation recovered from 1.125 to 2.93 log<sub>10</sub> CFU/mL during the storage period. NT-A-ref, being a combination of nisin and thymol demonstrated greater antilisterial efficacy compared to individual treatments N-A-ref and T-A-ref (except for 30 min incubation time), suggesting a minor collaborative effect between nisin and thymol in the water phase. On the other hand, bacterial population treated with formulation N-A-model and T-A-model (aqueous preparations of nisin or thymol) showed fastest recovery of cells to the negative control level after 3 days of incubation with juice, indicating strong deleterious effect of depletion factors. NT-A-model

formulation also recovered to the untreated control level after 5 days of storage (Figure 4.7b).

#### 4.4.5.2 *Listeria monocytogenes*: Emulsion systems

In the case of emulsions, colony count in N-E-ref and T-E-ref increased from 2.2 and 1.3 to 2.78 and 2.98 log<sub>10</sub> CFU/mL, respectively, whereas NT-E-ref showed an increase from 1.43 to 2.70 log<sub>10</sub> CFU/mL (Figure 4.7c and d). For all days of incubation, antilisterial efficacy due to NT-E-ref was statistically similar to NT-A-ref and significantly different from all other formulations ( $p < 0.05$ ).

#### 4.4.5.3 *Salmonella* Typhimurium: Non-emulsion systems

Nisin formulation N-A-ref were ineffective in inhibiting *Salmonella* Typhimurium. *Salmonella* Typhimurium is a Gram-negative bacteria with the presence of lipopolysaccharides in its outer membrane. Formulation T-A-ref and NT-A-ref demonstrated strong antibacterial efficacy, the log<sub>10</sub> CFU/mL increased from 2.56 and 1.0 to 3.76 and 3.84 over the 11 day period (negative control: 6.32 to 7.69 log<sub>10</sub> CFU/mL) (Figure 4.8a and b). Similar to the reference group, formulation N-A-model failed to demonstrate antibacterial activity against *Salmonella*, being a Gram negative bacteria. *Salmonella* log<sub>10</sub> CFU/mL count in T-A-model and NT-A-model recovered to buffer-model level after 3 days of incubation with juice, indicating the rapid depletion of thymol and nisin due to evaporation and proteolysis, respectively (Figure 4.8b).

#### 4.4.5.4 *Salmonella* Typhimurium: Emulsion systems

NT-E-ref treatment was the most effective, recovering from 1.1 to 2.9 log<sub>10</sub> CFU/mL over the 11 day period ( $p < 0.05$ ), suggesting that the combination of nisin and thymol showed the strongest antimicrobial activity for a prolonged time (Figure 4.8 c and d). NT-E-ref and T-E-ref formulations were statistically similar but significantly different from all other treatments ( $p < 0.05$ ). On the other hand, emulsion treatments demonstrated the activity prolongation effect in the presence of depletion factors. For example, NT-E-model demonstrated the greatest antibacterial efficacy (Figure 4.8d). It was significantly different from all other formulations ( $p < 0.05$ ) and demonstrated 2.8

$\log_{10}$  CFU/mL difference with the negative control after 7 days of incubation (Figure 4.8d).

#### 4.4.6 Emulsion as a protection and delivery tool for antimicrobials

##### 4.4.6.1 Nisin alone

Starch-OS stabilized emulsion system could prolong the antibacterial efficacy of nisin against *Listeria monocytogenes* V7. For example, in the model system N-E-model formulation demonstrated 3.76 (30 min) to 1.03 (5 days)  $\log_{10}$  CFU/mL reduction. N-E-ref formulation always demonstrated more than 3.5  $\log_{10}$  CFU/mL reduction against *Listeria monocytogenes* V7 with the untreated control on all days of incubation. Nisin combined with starch-OS emulsion was ineffective against *Salmonella* Typhimurium growth on all days of interaction.

##### 4.4.6.2 Thymol alone

T-E-model formulation recovered from 1.92  $\log_{10}$  CFU/mL to the untreated control level of *Listeria* in 7 days of incubation. In the reference group, T-E-ref showed strong antilisterial activity, the  $\log_{10}$  CFU/mL count increased from 1.3 to 2.98 over the 11 days of storage. For *Salmonella*, there was a gradual increase in  $\log_{10}$  CFU/mL count from 1.32 (30 min) to 6.41 (11 days) over the entire period of storage. Similar to the *Listeria* group, T-E-ref also demonstrated strong antibacterial efficacy against *Salmonella* Typhimurium over all the days of interaction with heat treated cantaloupe juice.

##### 4.4.6.3 Combination of nisin and thymol

Combined emulsion formulations of nisin and thymol, NT-E-model and NT-E-ref demonstrated the best antibacterial efficacy against both *Listeria* and *Salmonella* for a prolonged period of time. In the model group, NT-E-model formulation could show 2.53  $\log$  CFU/mL difference after 7 days of storage with cantaloupe juice, whereas NT-E-ref showed more than 3  $\log$  CFU/mL difference on all days ( $p < 0.05$ ). Similar trend in efficacy was also demonstrated by NT-E-model and NT-E-ref formulation against *Salmonella*. NT-E-model showed a 2.8  $\log$  CFU/mL difference whereas NT-E-ref mostly inhibited cells below 3.0  $\log$  CFU/mL for all days of storage.

#### 4.5 Discussion

The purpose of this study was to prolong the antimicrobial efficacy of two different biological compounds, nisin and thymol using an oil-in-water emulsion system. Figure 4.9 depicts a schematic comparison of the depletion, protection and antimicrobial efficacy of nisin, thymol and their combination against model organism using emulsion and aqueous solutions. In the presence of depletion factors, the available molecules in both the systems undergo gradual degradation. This degradation rate of the available antimicrobial is proportional to, or at least positively correlated to the concentration of non-available molecules in the aqueous system. In the presence of negatively charged bacterial cell membrane, a trigger mechanism is established that drives further release of the antimicrobial. Therefore, in order to reach equilibrium, there is a continuous re-distribution of molecules among the oil droplets and the interface, emulsion aqueous phase and the bacterial cell membrane.

The transfer of antimicrobial compounds in a food system depends greatly on its concentration difference between a “depot” and “sink” and the time of flow. In our antimicrobial loaded, starch-OS emulsion system, the oil droplets and interfacial layer served as thymol and nisin depot, respectively. Depletion of thymol and nisin can be associated due to presence of multiple sinks: (a) cantaloupe juice protease for nisin depletion, (b) chamber headspace for thymol evaporation, and (c) negatively charged bacterial cell membrane for antimicrobial complexation.

Initially, in the absence of bacterial cells, sinks (a) and (b) dominate the dynamics of antimicrobial depletion. Nisin is degraded by the action of proteolytic enzymes from cantaloupe juice (sink-a). Proteolytic enzymes have been commonly found in fruits, such as the alkaline serine protease cucumisin in melons (e.g. honeydews and cantaloupes), thiol protease actinidin in kiwi fruit, and cysteine protease bromelain in pineapples (Katsaros, Katapodis, & Taoukis, 2009; Raimbault, Zuily-Fodil, Soler, Mora, & Cruz de Carvalho, 2013; Yamagata, Masuzawa, Nagaoka, Ohnishi, & Iwasaki, 1994). While the major function of such proteases ranges from peptic cleavage to comprehensive proteolysis, they can significantly degrade nisin. Thymol, on the other hand, owing to its

low melting temperature is highly volatile under ambient conditions of storage.

Therefore, presence of experimental headspace drives thymol depletion.

After inoculation, bacterial cells acts as the major sink (sink-c) for antimicrobial motion from the droplet and interface depot. Nisin flows to the sink possibly due to electrostatic forces between positively charged nisin molecules and negatively charged cell membrane. Similarly, thymol motion from droplet depot to the sink is driven by possible hydrophobic interactions between thymol molecule and the phospholipid bilayer. At this point of time, the emulsion depot system shows its efficacy by the sufficient retention of active ingredients previously. Without the emulsion, both nisin and thymol would be substantially degraded by the action of depletion factors. As highlighted by our antibacterial efficacy data, the use of starch-OS emulsion prolonged antimicrobial efficacy after 7 days of incubation with cantaloupe juice, whereas the aqueous solutions of nisin and thymol ceased to show efficacy after 1-3 days of interaction.

Figures.4.9a, 4.9b and 4.9c depicts a schematic comparison of the depletion, protection and antimicrobial efficacy of nisin, thymol and their combination against a Gram-positive and Gram-negative model organism using emulsion and aqueous solutions. The Gram-positive bacteria was *Listeria monocytogenes* and Gram-negative organism was *Salmonella* Typhimurium. Nisin, being an antimicrobial peptide is effective mainly against Gram-positive bacterial cells such as *Lactococcus*, *Streptococcus*, *Staphylococcus*, *Micrococcus*, *Pediococcus*, *Lactobacillus*, *Listeria* and *Mycobacterium*, but ineffective against Gram-negative bacteria, yeasts and molds (de Arauz, Jozala, Mazzola, & Penna, 2009). Thymol, on the other hand is a derivative of cymene and consists of a phenolic hydroxyl group, a methyl group and an isopropyl group (Yanishlieva, Marinova, Gordon, & Raneva, 1999). Thymol demonstrates strong antibacterial and antifungal activity in bacteriological media and food systems (Burt, 2004). Therefore, a combination of nisin and thymol was exploited in order to generate enhanced antimicrobial efficacy for a prolonged period of time by incorporation with an emulsion based delivery vehicle.

The comparison between nisin protection, depletion and antibacterial effect is shown in Figure.4.9a. Scenario 1 demonstrates high stability of nisin in aqueous and

emulsion formulations at the initial starting point. In scenario 2, when the aqueous and emulsion preparations are added in cantaloupe juice, proteolytic activity from juice protease starts to drive nisin depletion. But starch-OS stabilized emulsion demonstrates the protective effect on nisin activity. In the emulsions, there is depletion of the aqueous phase nisin but a residual nisin concentration remains protected due to strong adsorption at emulsion interface. Scenario 3 demonstrates emulsion behavior in the presence of bacterial cells. Due to the presence of negatively charged bacterial cell membrane, a trigger mechanism is established that drives further release due to positively charged nature of nisin.

Figure 4.9b is a highlight of thymol depletion and protection in the presence of delivery systems and bacterial cells. Initially, thymol molecules reside inside the oil droplets, but with the progress of time, thymol is released into the water phase. The availability of thymol in the aqueous phase is governed by several factors such as its partition coefficient, interfacial thickness, pH of water phase and temperature. Scenario 1 demonstrates the initial stage of thymol spatial distribution in aqueous and emulsion formulations. Thymol is partitioned from the oil to the water phase due to diffusive mass transfer phenomenon. In scenario 2, there is rapid depletion of thymol due to evaporation in the aqueous solution, indicating the effect of chamber headspace. In the emulsion group there is depletion of aqueous phase thymol due to evaporation, but the residual amount being still protected inside the oil droplets. In scenario 3, the negligible concentration of thymol in the aqueous phase binds with bacterial cells thereby showing minor antibacterial efficacy. In the emulsion group, there is a continuous release of thymol molecules from the droplet interior across the interface. Therefore, aqueous thymol concentration is still sufficient to demonstrate stronger antibacterial efficacy.

Figure 4.9c is a schematic summary of the combined effects of nisin and thymol with and without the delivery system. Scenario 1 aqueous solution group depicts the initial stage where both nisin and thymol molecules are dispersed in sodium acetate buffer. The emulsion group shows that nisin is distributed among the interface and aqueous phase whereas thymol is partitioned between oil droplets and emulsion water phase. Scenario 2 demonstrates the dynamics of the systems in the presence of cantaloupe



juice protease and chamber headspace as depletion factors. In the non-emulsion, there is rapid depletion of nisin due to proteolytic activity and thymol due to evaporation, thereby reducing their effective concentrations drastically. In the emulsion, the delivery system provides protection to the antimicrobial molecules due to adsorption at interface (nisin) and hydrophobic interactions inside oil droplets (thymol). A certain concentration of the total antimicrobials is available in the water phase that inhibits the growth of bacterial cells in the next scenario. Scenario 3 is the most complicated situation where both the antimicrobials are available in the aqueous phase for antibacterial efficacy. Nisin and thymol places themselves on the cell surface; 8 nisin molecules forms pore like structures, whereas thymol uses the hydroxyl group in its phenolic structure to enhance cellular permeability.

#### 4.6 Conclusion

In summary, our current research demonstrated the potential of using starch-OS based materials for the simultaneous protection of two antimicrobials (nisin and thymol) against depletion factors for the inhibition of *Listeria* and *Salmonella*. Starch-OS stabilized emulsion system substantially improved thymol and nisin activity against different depletion factors to achieve prolonged protection, whereas the non-emulsion systems showed rapid signs of depletion. The strongest antibacterial efficacy for a prolonged time in the model system was demonstrated by formulation NT-E-model, showing 2.53 log<sub>10</sub> CFU/mL and 2.80 log<sub>10</sub> CFU/mL difference with the negative control after 7 days of depletion. Nisin and thymol demonstrated a collaborative action regarding inhibition of bacterial cells in both aqueous and emulsion formulations. In addition, thymol and nisin partitioning in the emulsion water phase was the key driving force behind prolonged antibacterial efficacy. Partitioning experiments confirmed that thymol remained mostly associated with the emulsion lipid phase with partial release into the aqueous phase over time. Therefore, starch-OS based oil/water emulsion systems shows

great potential regarding better protection and activity retention of peptides and essential oil components as antimicrobials in fresh-cut produce.

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Table 4.1 Summary of formulations in Model and Reference group

Formulation Nomenclature	Types of formulation	Environment	
		Model group (Cantaloupe juice and headspace)	Reference group (Heat-treated cantaloupe juice and no headspace)
N-A-model	Nisin in aqueous solution	✓	
N-A-ref			✓
T-A-model	Thymol in aqueous solution	✓	
T-A-ref			✓
NT-A-model	Combination of nisin and thymol in aqueous solution	✓	
NT-A-ref			✓
N-E-model	Nisin in emulsion	✓	
N-E-ref			✓
T-E-model	Thymol in emulsion	✓	
T-E-ref			✓
NT-E-model	Combination of nisin and thymol in emulsion	✓	
NT-E-ref			✓
Buffer-model	Negative control (sodium acetate buffer)	✓	
Buffer-ref			✓



Table 4.2 Mean partition coefficients ( $P_{o/w}$ ) of thymol in different emulsion preparations over 11 days of storage.

Formulation	Thymol only emulsion- Model group	Thymol + nisin emulsion- Model group	Thymol only emulsion- Reference group	Thymol + nisin emulsion- Reference group
Time	$P_{o/w}$	$P_{o/w}$	$P_{o/w}$	$P_{o/w}$
30 min	$1.45 \pm 0.34^a$	$1.40 \pm 0.64^b$	$1.89 \pm 0.56^c$	$1.39 \pm 0.31^d$
1 day	$1.55 \pm 0.62^a$	$1.45 \pm 0.52^b$	$2.32 \pm 0.75^c$	$2.77 \pm 0.65^d$
3 day	$1.69 \pm 0.29^a$	$1.65 \pm 1.57^b$	$2.26 \pm 0.43^c$	$1.81 \pm 0.62^d$
5 day	$2.14 \pm 1.99^a$	$1.37 \pm 1.33^b$	$1.90 \pm 0.18^c$	$1.94 \pm 0.82^d$
7 day	$1.21 \pm 0.51^a$	$1.42 \pm 0.70^b$	$2.18 \pm 0.30^c$	$2.06 \pm 0.88^d$
11 day	$2.72 \pm 1.44^a$	$0.97 \pm 1.36^b$	$2.04 \pm 0.50^c$	$2.00 \pm 0.82^d$

Mean values in each column with similar letters are statistically not significant ( $p > 0.05$ ).

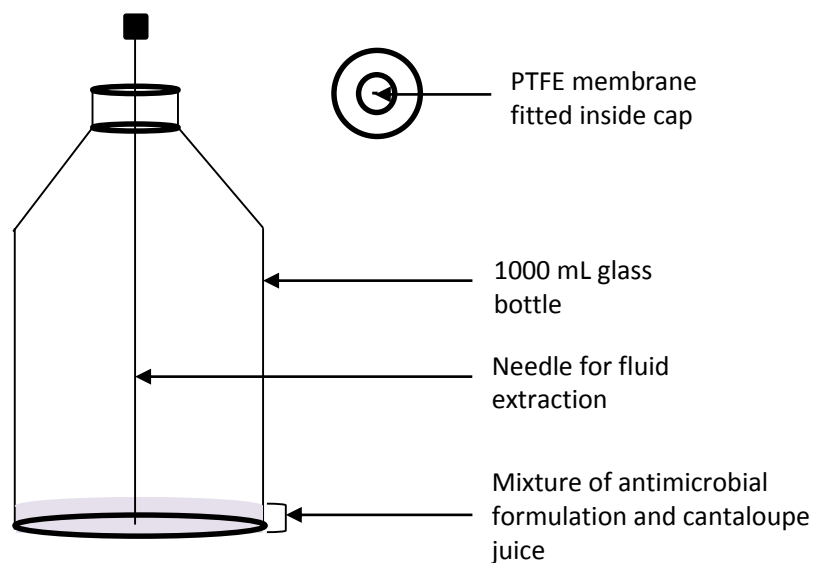


Figure 4.1 “Model” group experiment was performed using a 1000 mL glass bottle. The total volume of fluid in the bottle was 50 mL with a headspace of 950 mL. A 12” long needle was used to extract the fluid after 30 min, 1, 3, 5, 7, and 11 days of interaction with fresh cantaloupe juice. The cap of the bottle was fitted with a PTFE membrane for sample extraction without opening.

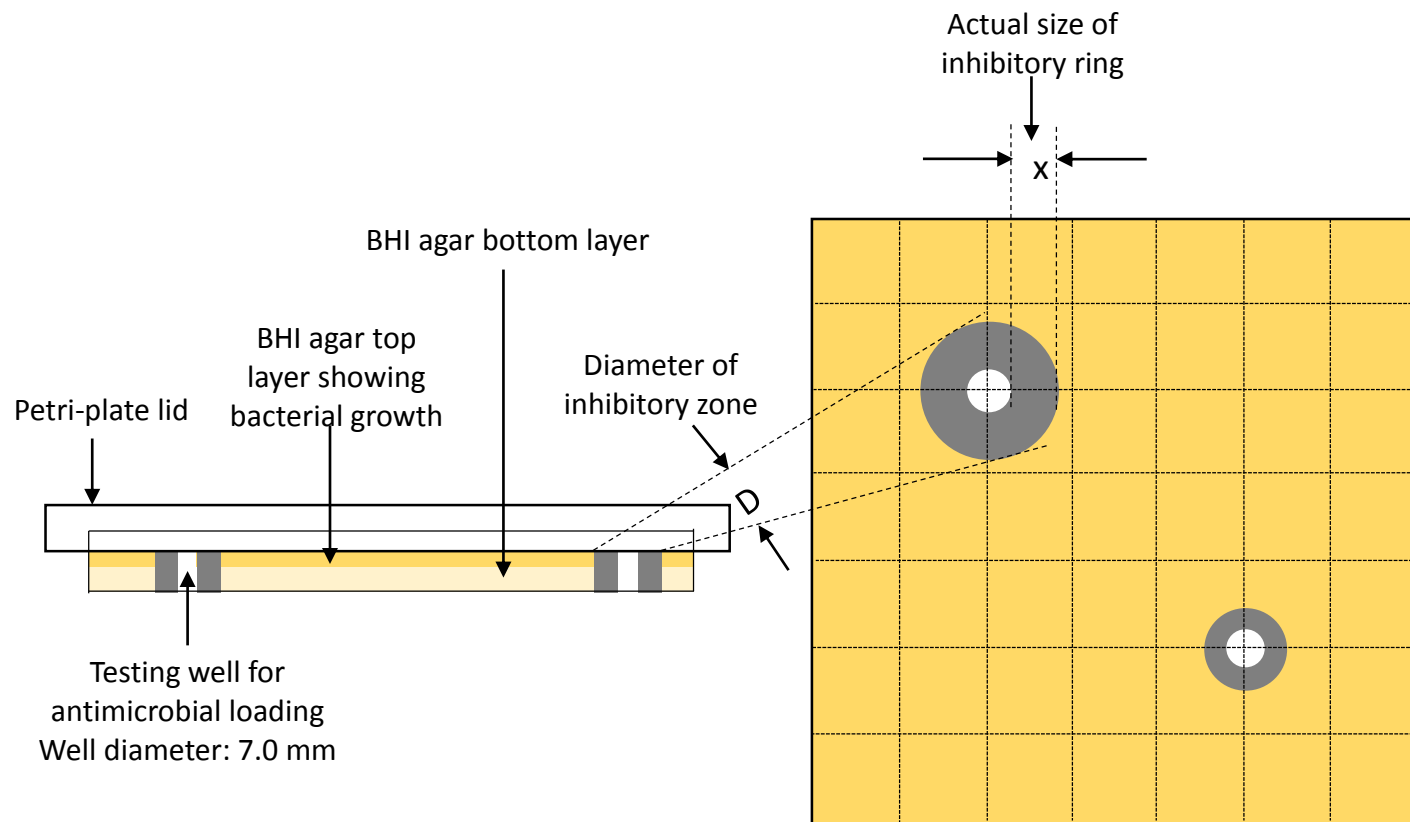


Figure 4.2 Schematic of agar diffusion bioassay for nisin quantification process. Left figure: Side view of a square petri-plate is shown. The top layer of BHI-agar shows the growth of *Listeria monocytogenes*. The bottom layer comprises of BHI agar. Right figure: Top view of a square petri-plate is shown with its grid lines. The transparent zone indicates the inhibitory effect of nisin on bacterial growth. D is the diameter of the inhibitory zone and x is the actual size of the inhibitory ring, where  $x = (D-7)/2$

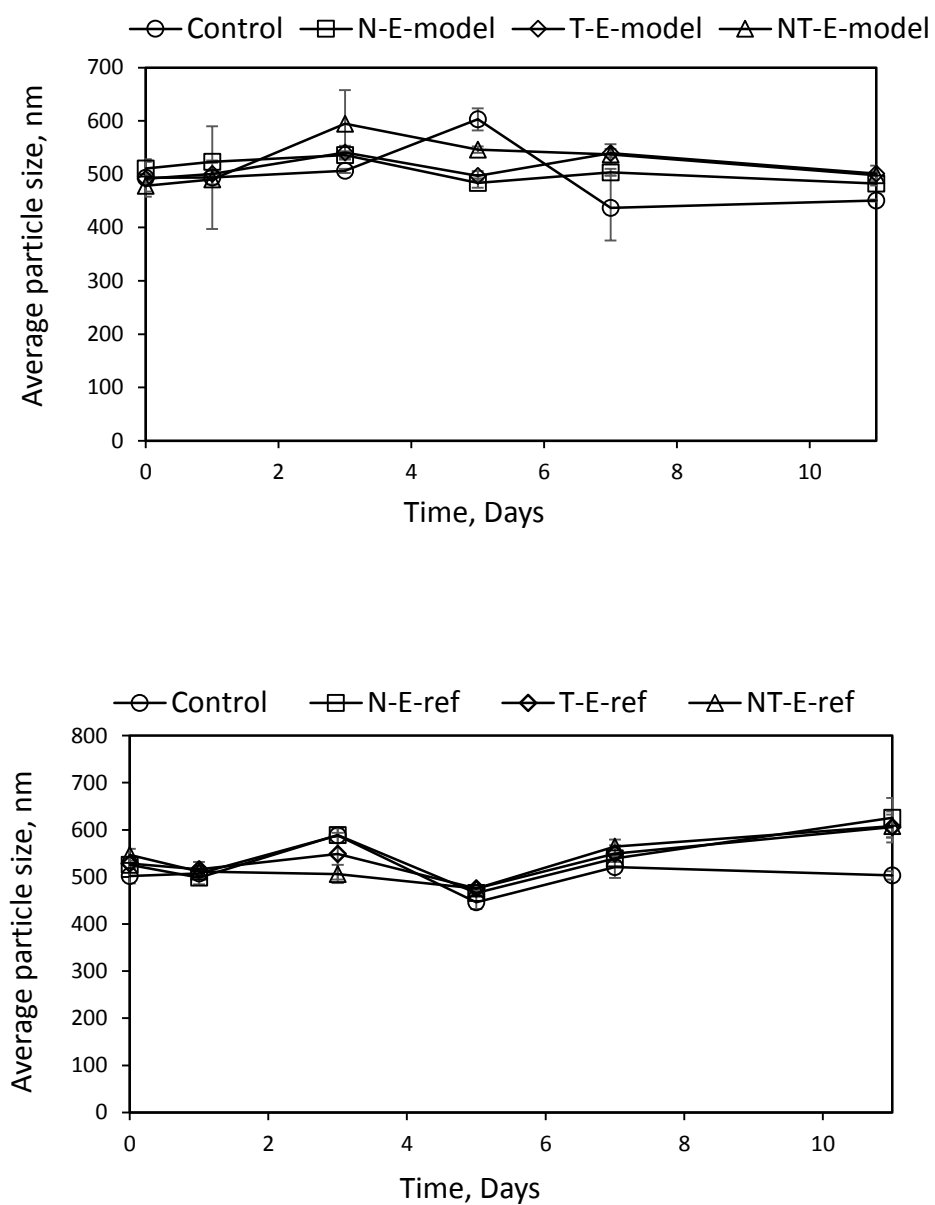


Figure 4.3 Average particle size for different emulsion formulations in the model and reference groups. Mean values are shown with error bars of standard deviations (n= 3).

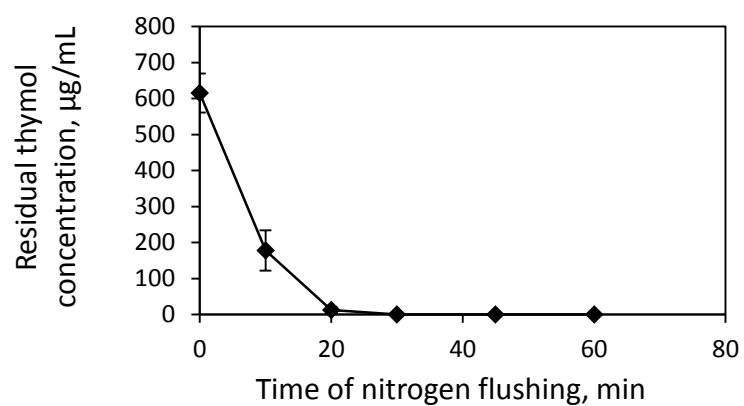
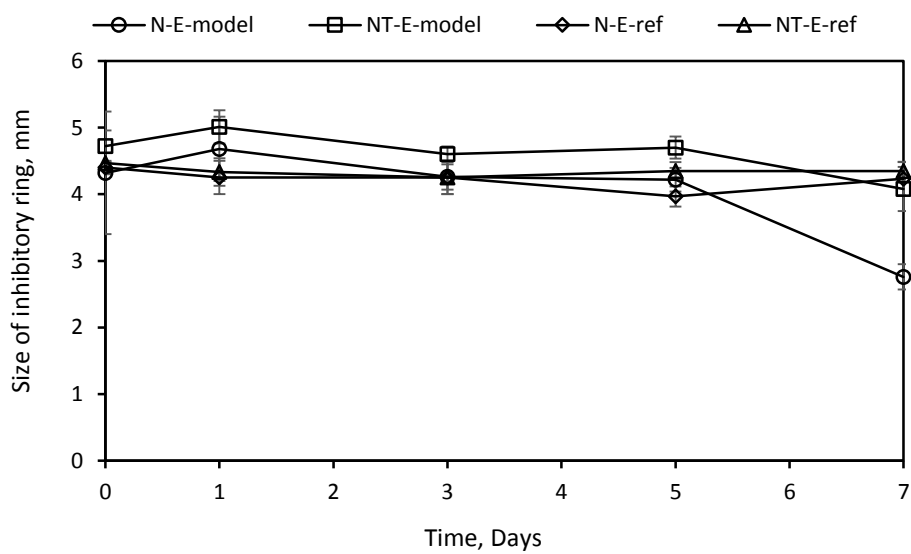
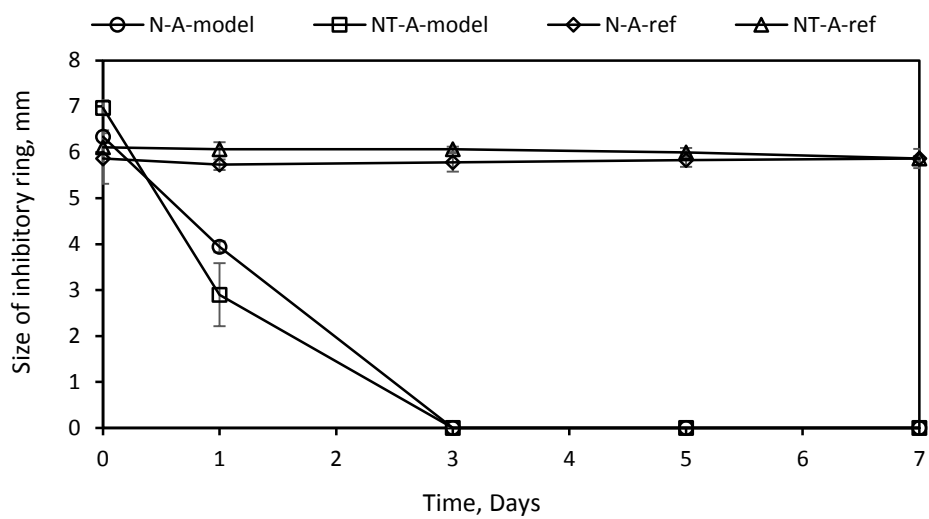


Figure 4.4 Reduction of thymol concentration in sodium acetate buffer (50 mM, pH 5.5) after nitrogen flushing



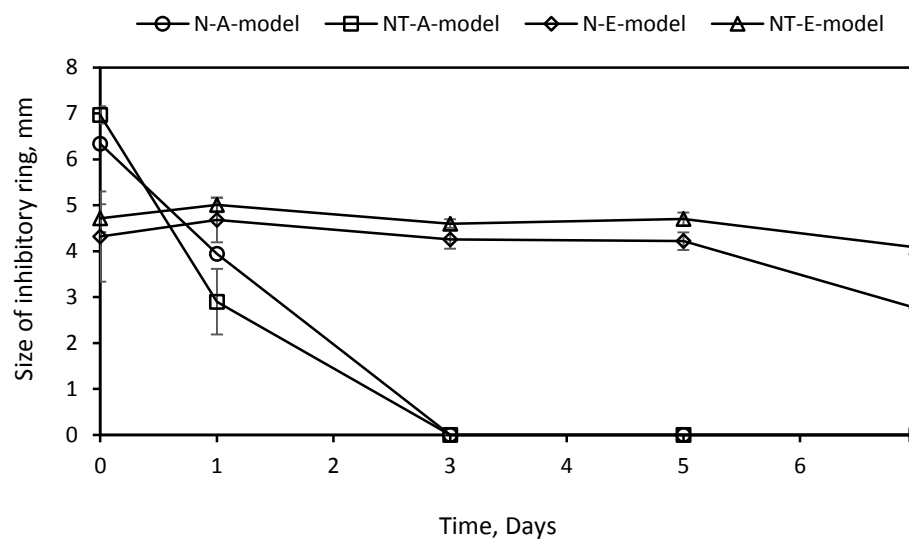
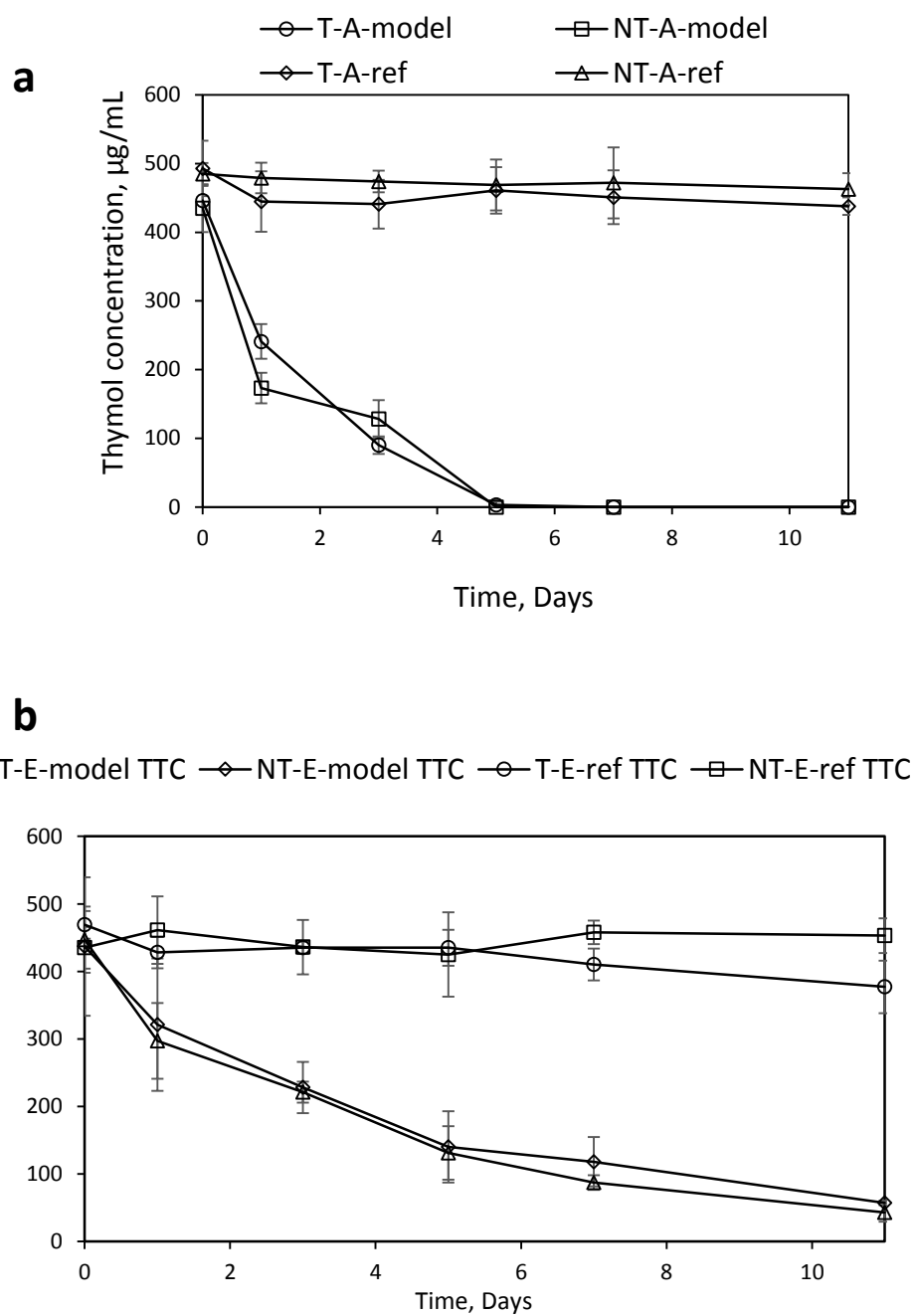


Figure 4.5 (a) Zone of inhibitory ring for aqueous (non-emulsion) systems is plotted versus time for both model and reference groups, (b) Zone of inhibitory ring for emulsion systems is plotted versus time for both model and reference groups, (c) Zone of inhibitory ring for non-emulsion and emulsion systems for model groups are plotted. Time point “0 days” indicate 30 min of interaction with model or reference group. Mean values are shown with error bars of standard deviations (n= 3).





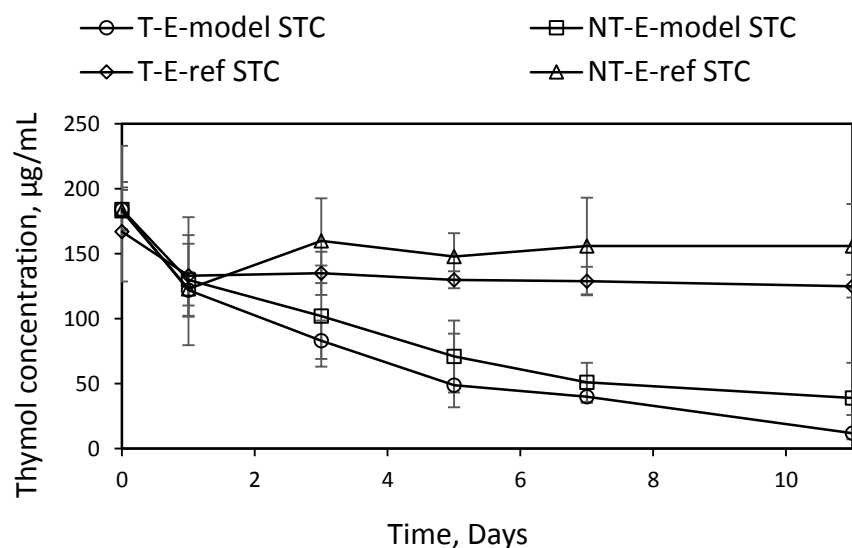
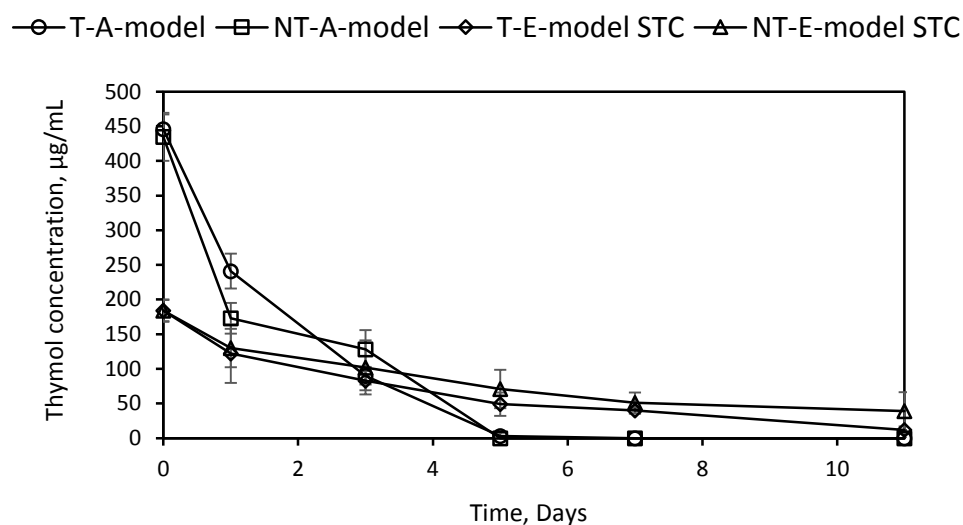
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Figure 4.6 (a) Thymol concentration in aqueous (non-emulsion) systems is plotted versus time for both model and reference groups, (b) Total thymol concentration in emulsion systems is plotted versus time for both model and reference groups, (c) Serum thymol concentration in model and reference groups are plotted for emulsion systems, (d) Comparison between thymol concentration in non-emulsion versus serum thymol of emulsion systems in model groups. Each emulsion formulation shows total thymol concentration (TTC) and serum thymol concentration (STC). Time point “0 days” indicate 30 min of interaction with model or reference group. Mean values are shown with error bars of standard deviations ( $n=3$ ).

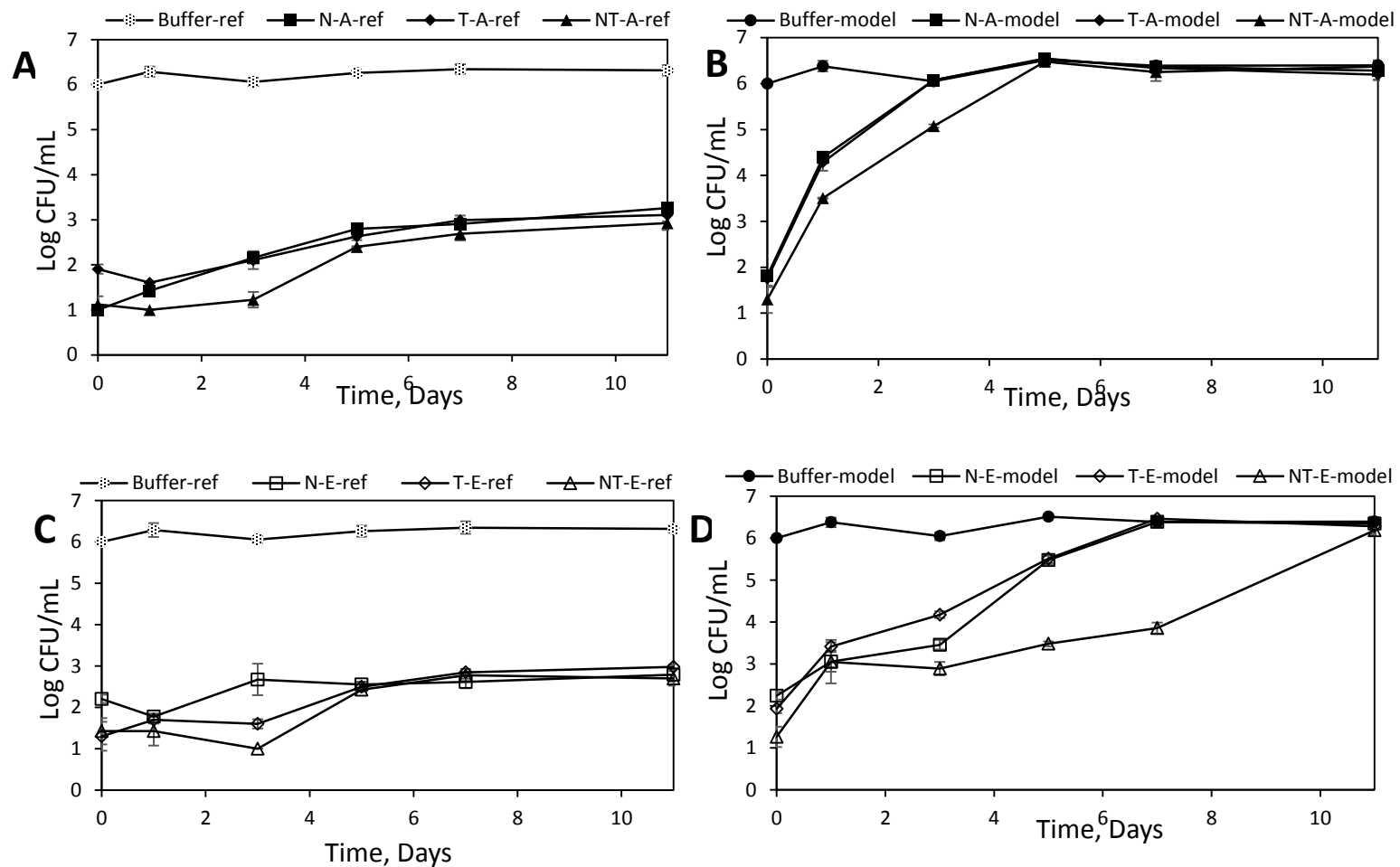


Figure 4.7 (A) and (B) *Listeria monocytogenes* V7 log CFU/mL count versus time for aqueous (non-emulsion) system is show for reference and model groups, respectively. (C) and (D) *Listeria monocytogenes* V7 log CFU/mL count versus time for emulsion system is shown for reference and model groups, respectively. Time point “0 days” indicate 30 min of interaction with model or reference group. Mean values are shown with error bars of standard deviations (n= 3).

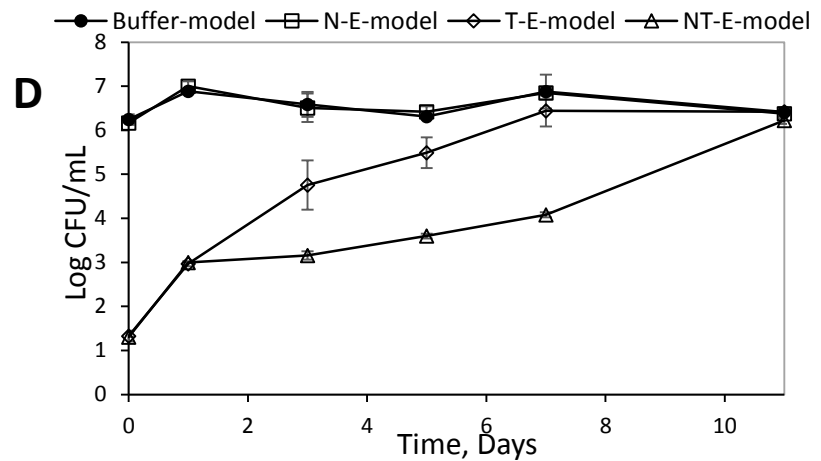
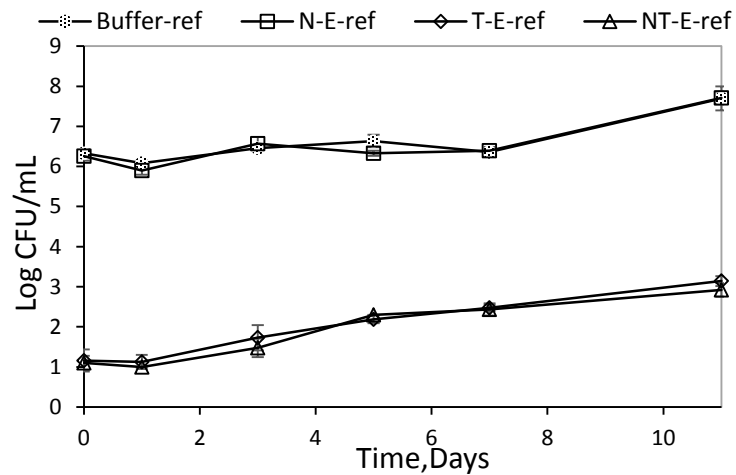
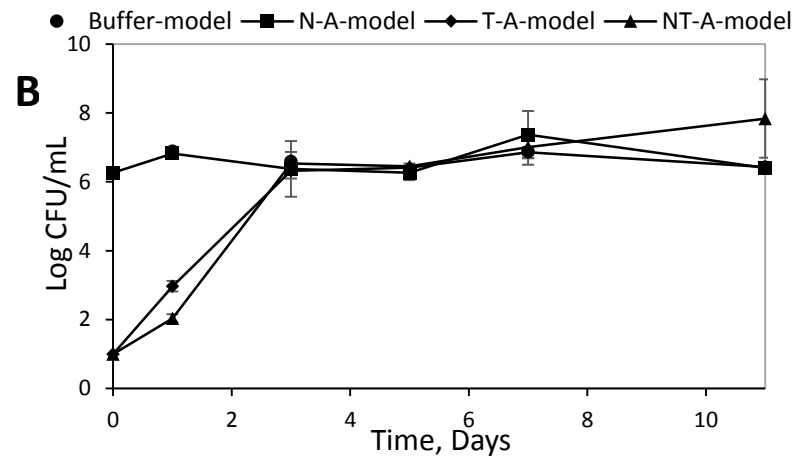
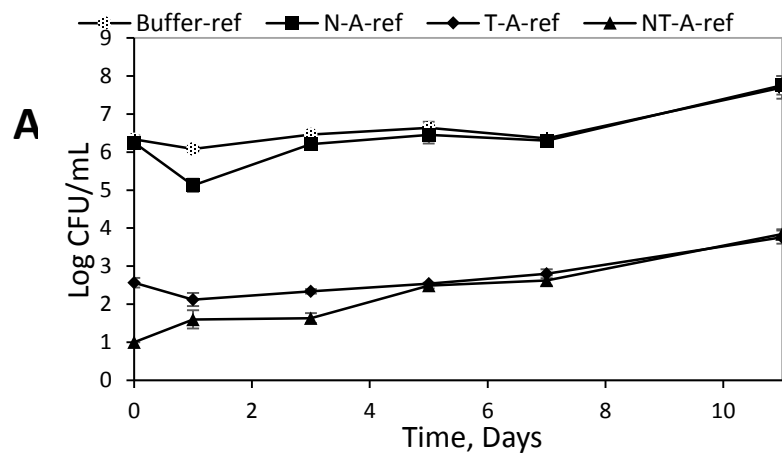


Figure 4.8 (A) and (B) *Salmonella* Typhimurium log CFU/mL count versus time for aqueous (non-emulsion) system is show for reference and model groups, respectively. (C) and (D) *Salmonella* Typhimurium log CFU/mL count versus time for emulsion system is shown for reference and model groups, respectively. Time point “0 days” indicate 30 min of interaction with model or reference group. Mean values are shown with error bars of standard deviations (n= 3).

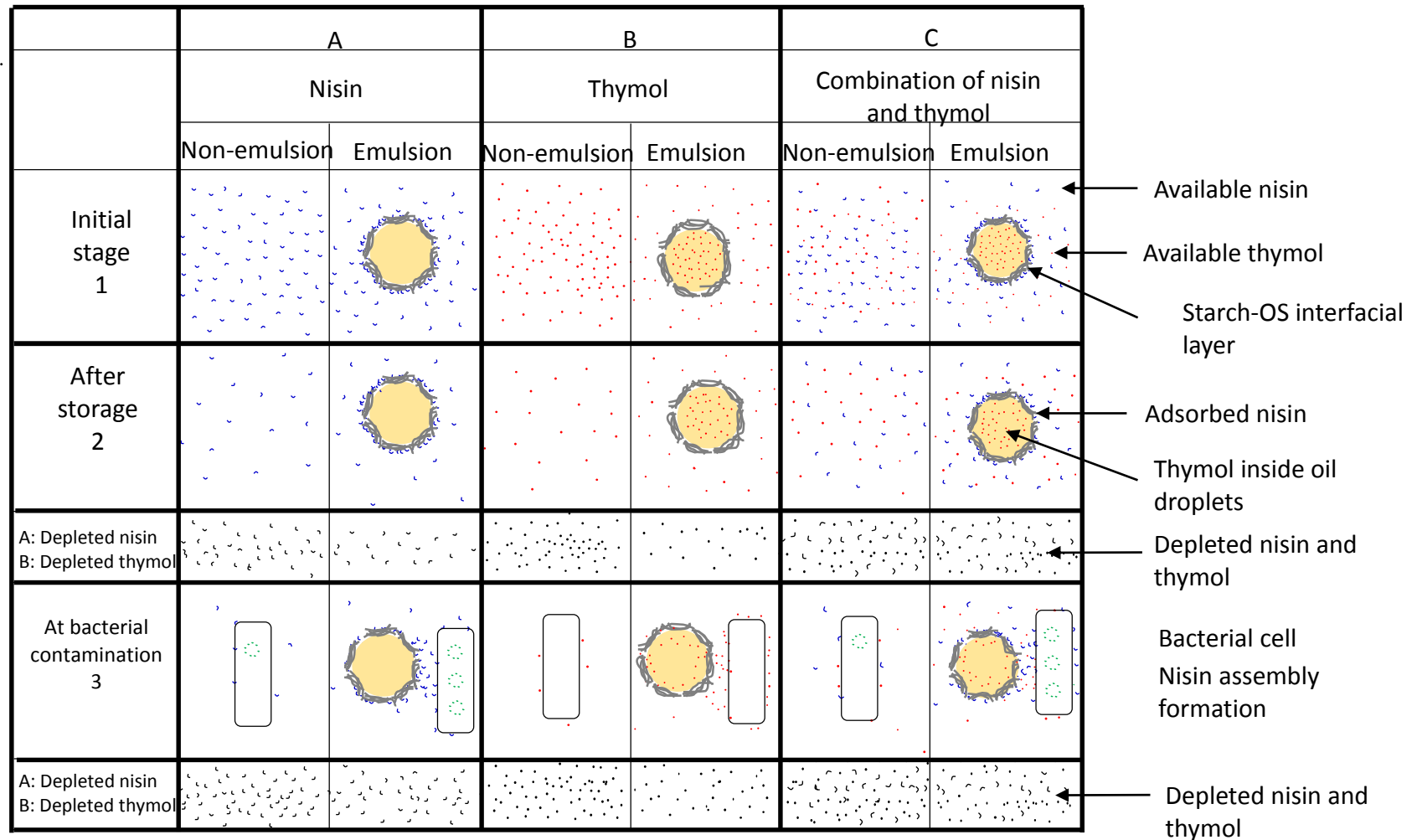


Figure 4.9 Schematic representation of the availability, depletion, protection and antibacterial efficacy of nisin, thymol and a

## CHAPTER 5. OVERALL CONCLUSIONS AND FUTURE RECOMMENDATIONS

The present dissertation was an attempt to study the relationships between antimicrobial peptides and phenolics and carbohydrate based biopolymeric systems. The first part of the work was performed to understand interactions between antimicrobial peptides and carbohydrate based nanomaterials in two types of dispersion systems, non-emulsion (aqueous) and emulsions. Nisin was used as the model antimicrobial peptide and phytoglycogen octenyl succinate (PG-OS) was the model carbohydrate biopolymer. The study established that the degree of substitution (DS) of PG-OS materials was the key factor driving nisin adsorption in the non-emulsion (aqueous) phase. Adsorption of nisin to PG-OS in both non-emulsion and emulsion phases followed a Langmuir monolayer adsorption pattern. The Langmuir equation was used to calculate adsorption parameters including the monolayer adsorption capacity ( $Q_m$ ) and equilibrium constant for adsorption ( $K$ ). Data revealed that the monolayer adsorption capacity or ( $Q_m$ ) value increased with an increase in DS of PG-OS in the non-emulsion system, suggesting the increased availability of OS binding sites on phytoglycogen (PG) surface. On the other hand, the DS of PG-OS only slightly affected the equilibrium constant ( $K$ ) value. In the case of emulsions, even the lowest DS of PG-OS material showed a higher  $Q_m$  value compared to the highest DS of PG-OS in aqueous phase. This significant increase in  $Q_m$  value demonstrates the greater adsorption potential of nisin to PG-OS stabilized interfacial layer. In addition, at equivalent concentrations of PG-OS and nisin, the adsorbed nisin concentration in emulsion was seven times higher than non-emulsion phase.

PG-OS stabilized emulsion showed lower K value compared to the K value of equivalent PG-OS material in the non-emulsion phase. K value was related to the adsorption enthalpy ( $\Delta H$ ) through the Clausius-Clayperon equation. We proposed that this strong interaction between nisin and PG-OS colloidal assembly is possibly due to both electrostatic and hydrophobic interactions. In addition, the lower K value and a lower  $\Delta H$  value also indicates that nisin possibly interacts with the oil droplet surface due to weak hydrophobic interactions.

In the second part of the study, nisin was combined with starch octenyl succinate (starch-OS) emulsion and the protection capability was evaluated in a cantaloupe juice model against *Listeria monocytogenes*. Cantaloupe juice was selected as the model depletion system because of the significant presence of protease enzymes which depletes nisin. The study confirmed that protease in cantaloupe juice was the key factor responsible for nisin depletion. In addition, starch-OS stabilized emulsion could prolong nisin activity till six days of interaction with juice components, whereas the activity in aqueous nisin dispersion was lost after 2 days of interaction. Equilibrium dialysis experiments demonstrated that adsorption between nisin and starch-OS colloidal assembly was the driving force behind prolonged nisin activity against *Listeria monocytogenes*. In the third part of the thesis, nisin and thymol were combined with starch-OS based delivery vehicle and their prolonged antimicrobial efficacy was evaluated against both *Listeria monocytogenes* and *Salmonella* Typhimurium. Cantaloupe juice was used as the depletion system for nisin and chamber headspace for thymol depletion. Starch-OS colloidal assembly could prolong the activity of nisin and thymol against both *Listeria* and *Salmonella* till seven days of storage, whereas the aqueous solutions of antimicrobials lost their efficacy after three days. Minor collaborative effect regarding antimicrobial efficacy was observed between nisin and thymol. The best antibacterial efficacy for a prolonged period of time was demonstrated by the emulsion formulation which combined both nisin and thymol. In addition, partitioning studies demonstrated that thymol remained mostly associated with the lipid phase of the emulsion.

In the context of the first study, the use of PG-OS with DS other than 0.010 should be evaluated for nisin adsorption in the emulsion system. Similar equilibrium dialysis technique can be applied for understanding the nisin adsorption phenomenon. In addition, enzymatic treatment with beta-amylase to PG-OS might enhance nisin adsorption by the creation of physical spaces on its structure. In the second study, the use of PG-OS in protecting the effect of nisin should be evaluated in the presence of cantaloupe juice components.

With regards to the study of nisin and thymol protection, the potential of PG-OS with DS of 0.01 shall be compared with starch-OS. PG-OS being a hyper-branched, dendrimer like molecule, might be able to extend the effect of both nisin and thymol in the presence of depletion factors. In addition, the incorporation of thymol within PG-OS nanoparticles can be explored. The release and collaborative effects between PG-OS encapsulated thymol and nisin can then be studied against model Gram-positive and negative organisms.

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